THE ESSENTIAL ROLE OF FATTY ACIDS IN RATIONS FOR GROWING CHICKS[1]

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ONE FIGURE

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Data on the requirement of growing chicks for fat in their rations are meager (Russell et al., '40). As has been recently reported (Reiser, '50a), most studies on the fat requirement of poultry have been made on laying hens. The bulk of the evidence indicates that fat is not required for production, hatchability or growth.

EXPERIMENTAL

In the present study, White Leghorn cockerels were fed a basal fat-free ration alone or supplemented with cottonseed oil, lard, recrystallized bayberry tallow, or methyl palmitate. The basal ration is given in table 1. In some tests tocopherol acetate was used. Later it was found that a commercial preparation of mixed tocopherols[2] contained no polyunsaturated fatty acid that could be identified by alkaline isomerization, and this product was then substituted for the pure ester. The fat soluble vitamins were mixed with the test fat or Tween 20.[3] In some tests they were mixed in the ration; in others they were fed by dropper.

[1] This work was supported in part by grants from the Office of Naval Research and the Dow Chemical Company, under contracts with the Texas A. and M. Research Foundation.

[2] Distillation Products, Inc.

[3] Sorbitan monolaurate polyoxyalkylene derivative, the Atlas Powder Co.

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In each experiment there was one group on a stock ration and one on the fat-free ration. Twelve to 15 chicks were started in each group.

In table 2 is given a summary of the growth rates in all experiments. It is immediately obvious that in the absence of linoleic acid chicks fail to grow at a rate comparable to that of those receiving commercial mash or a fat supplement containing linoleic acid. Either 1% or 4% cottonseed oil or lard, when

TABLE 1

Basal fat-free ration

	%		%
Sucrose	52.8	Methionine	0.8
Soybean protein [1]	35.0	Glycine	2.0
Liver L [2]	4.0	Inositol	0.125
Salts	5.0	Choline	0.25
	mg/kg		*mg/kg*
Thiamine	6.0	Biotin	0.3
Ca pantothenate	25.0	*p*-Aminobenzoic acid	3.0
Niacin	70.0	Folic acid	2.5
Pyridoxine	8.0	Riboflavin	4.0

	mg/kg [3]	*mg/chick/day* [4]		*mg/kg*	*mg/chick/day*
Carotene	40.0	2.0	Vitamin D_3 [5]	0.15	0.01
Menadione	3.3	0.2	Tocopherol	400	10.0

[1] "Alpha" protein, obtained from the Glidden Co., Chicago, Illinois.

[2] Obtained through the courtesy of the Wilson Laboratories, Division of Wilson and Company, Inc., Chicago, Illinois.

[3] When mixed with oil in the rations.

[4] When mixed with Tween 20 and fed as a supplement.

[5] Vitamin D_3 was generously donated by Du Pont de Nemours and Company.

added to the ration, resulted in gains of the same order as those produced by the mash.

Few experiments were continued as long as 4 weeks because, as is shown in table 2, there were few or no survivors on the fat-free or saturated fatty acid rations after three weeks. It was considered unnecessary, therefore, to continue the controls.

In some of the earlier tests the residual yolks were removed from the chicks on the possibility that their absence might

TABLE 2

Rate of gain of chicks on rations with and without fat

RATION	TOTAL NO. OF CHICKS	NO. OF EXPTS.	WEIGHED AVERAGE WEIGHT [1]				SURVIVORS			
			1 wk.	2 wk.	3 wk.	4 wk.	1 wk.	2 wk.	3 wk.	4 wk.
			gm	*gm*	*gm*	*gm*	%	%	%	%
Mash	99	6	63	100	166	189	95	95	92	88
1% CSO [2]	18	2	60	88	148		100	78	36	
4% CSO [2]	43	3	58	88	151	198	95	80	70	70
1% Lard	31	2	72	102	136		100	84	80	
4% Lard	31	2	80	121	142		100	87	68	
Fat-free	78	6	48	58	87	99	95	41	22	13
1% Methyl palmitate	14	1	68	46			94	36	0	
1% Bayberry tallow	16	1	41				100	0	0	

[1] Statistical analysis is difficult because of the lack of survivors in some trials at the end of the third and 4th weeks. The weighed average weights and the percentage of survivors were, therefore, used as the criteria of the relative adequacies of the ration.

[2] Cottonseed oil (Wesson).

deplete the birds more quickly of essential fatty acids. However, the chicks from which the yolks had been removed showed no handicap, the rates of gain being approximately the same as those for the unoperated groups.

In table 3 are shown the results of an experiment with chicks hatched from the eggs of hens which had been on a comparable fat-free ration for 15 weeks. The major difference in this ration was that it contained egg albumen instead of soybean protein. The fat in these eggs contained no hexaene or pentaene fatty acids; only traces of tetraene and approxi-

TABLE 3

Growth of chicks hatched from hens on a fat-free ration

CHICK NO.	DAY-OLD	1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS	31 DAYS
	gm	*gm*	*gm*	*gm*	*gm*	*gm*
			Wesson oil ration			
201	42	54	76	133	178	225
202	40	56	90	158	212	274
			Fat-free ration			
203	44	64	82	116	164	Died
204	44	55	74	80	107	150 [1]
205	35	44	60	92	103	134

[1] Extremely edematous and moribund. The increase in weight was doubtless due to the edema.

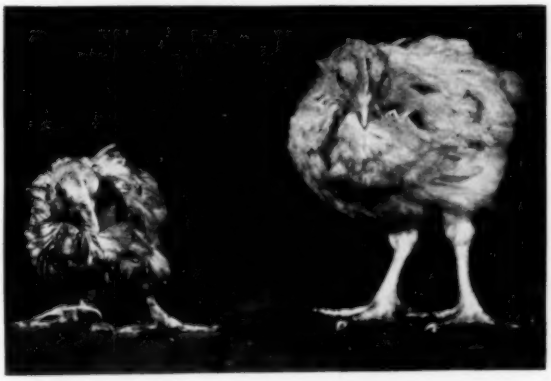
mately 0.2% triene and 1.5% diene. The chicks were fed the same ration as the hens, 2% cottonseed oil [4] being added to the control ration. Figure 1 is a photograph of chicks from these two groups at 4 weeks and three days of age. This experiment was part of a study of the effect of fatty acids in the ration on body fat (Reiser, '50b). Briefly, it was found that the total amounts of polyunsaturated fatty acids in the 4-week-old chicks were no greater than those in the eggs.

In all the experiments performed the fat-free groups invariably gained at a slower rate than those receiving some

[4] Wesson.

polyunsaturated fatty acid. That this effect is not one of fat alone may be concluded from the negative effect when bayberry tallow or methyl palmitate was added.

One peculiar symptom was noted. Frequently a moribund chick was observed to be extremely edematous, the subcutaneous layers having the appearance of a clear jelly, giving the chick an almost transparent effect. In one case the peritoneal cavity was filled with a thin jelly.



A B

Fig. 1 The chicks shown above are 4-week- and three-day-old White Leghorn, Rhode Island Red crosses hatched from eggs of White Leghorn hens which had been on a fat-free ration for 15 weeks. A received the same ration as the hens; B received 2% Wesson oil in addition to the basal ration.

CONCLUSION

Polyunsaturated fatty acids are essential nutrients in the growth of chicks.

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THE METABOLISM OF POLYUNSATURATED FATTY ACIDS IN GROWING CHICKS[1]

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There is reliable evidence that the fatty acid composition of poultry may be affected by the nature of the fat in the ration (Cruickshank, '34; Kummerow et al., '48; Hite et al., '49). However, beyond broad generalizations (Cruickshank et al., '39), little is known about the interrelations of the fatty acids within poultry tissue or the effects of specific fatty acids in the ration on the nature of tissue acids.

The requirement of the rat for polyunsaturated acids has received considerable attention, although it is still not completely settled whether or not this animal may be able to synthesize some polyunsaturated acids (Barki et al., '49).

Studies with other species are meager. Russell et al. ('40) fed growing chicks an extracted natural ration and found no difference in growth rate as compared to that obtained with an unextracted ration. These workers reported a lower iodine number in the depot fat of the chicks on the extracted ration but no difference in the liver fat.

Rieckehoff et al. ('49) have reported that tetraenoic acid may be synthesized from dienoic in the rat, and Widmer and Holman ('50) have also presented evidence that dienoic acid

[1] This work was supported in part by grants from the Office of Naval Research and the Dow Chemical Company of Midland, Michigan. A preliminary report was made at the meetings of the Federation of American Societies for Experimental Biology at Atlantic City, April 17–21, 1950 (Fed. Proc., *9:* 218, 1950).

results in the deposition of tetraenoic, and that trienoic may give rise to hexaenoic.

It has recently been reported from this laboratory (Reiser, '50) that on a fat-free ration hens deposit decreasing quantities of polyunsaturated fatty acids in their eggs, but that they can deposit increasing quantities of 4 and 5, but not 6, double bond acids upon the ingestion of linoleic acid fed as cottonseed oil. (At that time it was reported that two double bond acids were converted to three double bond acids, but work now in progress shows that this apparent effect was due to the spectral reflection of tetraenoic acid on absorption of 268 mμ.) The low-fat ration had no effect on production or hatchability or on the total fat or lipid partition of the eggs after 14 weeks.

The present studies were initiated to determine to what extent, if any, growing chicks can: (a) synthesize polyunsaturated fatty acid from non-fatty precursors; and (b) interconvert specific fatty acids of the ration.

Day-old White Leghorn or crossbred (Single Comb White Leghorn females × New Hampshire Red males) cockerels were fed the sucrose-egg albumen ration given in table 1. The high biotin level was included because of avidin in the albumen. Four-week-old chicks taken from the Experiment Station flock were used as controls. These chicks had been fed a commercial growing mash, which contained 4.36% total ether soluble extract.

In the test experiments, 2% recrystallized bayberry tallow, cottonseed oil,[2] triolein, ethyl linoleate, a mixture of ethyl linoleate and linolenate, or cod liver oil were substituted for 2% sucrose. Fifteen chicks were placed on each test ration and sacrificed after 4 weeks.

PREPARATION

Bayberry glycerides

Bayberry tallow was crystallized from 10 volumes of acetone. The product had an iodine value of three. The fatty

[2] Wesson.

acids had a mean molecular weight of 244 and contained no fatty acids with two or more double bonds.

Triolein

Oleic acid was prepared by the method of Swern et al. ('46).[3] The triglyceride was prepared from the acid by refluxing at a pressure of 2 mm of mercury with 90% of the theoretical

TABLE 1

BASAL FAT-FREE RATION

	Major constituents		
	%		%
Sucrose	68	Choline	0.25
Glycine	1.7	Salts 5	5
Albumen (egg)	25	Inositol	0.125
	Water-soluble vitamins		
			mg/kg
Thiamine	6 mg/kg	Niacin	70
Ca-Pantothenate	25 mg/kg	Folic acid	2.5
Pyridoxine	8 mg/kg	Biotin	5
Lederle's liver extract for B_{12}	6 ml/kg	Riboflavin	8
		p-Aminobenzoic acid	3
	Fat-soluble vitamins [1]		
Mixed tocopherols	1 gm/kg	Carotene	33 mg/kg
Menadione	1.5 mg/kg	Vitamin D_3	0.08 mg/kg

[1] Mixed with equal weight of Tween 20 and emulsified in water.

amount of glycerol and 0.5% naphthalene-β-sulfonic acid. The preparation contained no two or more double bond acid detectable by spectrophotometric methods.

[2] The author is indebted to Dr. R. R. King of Mrs. Tuckers Foods, Inc., Sherman, Texas, for the selective hydrogenation of the beef tallow from which this acid was prepared.

Linoleate

Ethyl linoleate was prepared by two different procedures, as follows:

(a) Cottonseed oil [4] was saponified according to the method described by Swern et al. ('45). The fatty acids were fractionally crystallized from acetone and the fraction soluble at — 55°C. in 10% concentration esterified with ethanol. The ester had an iodine number of 156 (Theory 165). It contained no fatty acids with more than two double bonds.

(b) To 500 gm of cottonseed oil were added 165 ml of absolute alcohol (commercial) to which had previously been added 3 gm of metallic sodium. The mixture was held at 50°C. for three hours, protected from atmospheric moisture. The reaction mixture was decanted from the precipitated glycerol and washed with hot water until clear. Ethyl esters of saturated and oleic acids were removed from the dried ester mixture by precipitation as urea complexes.[5] The product had an iodine value of 150.

Linoleate-linolenate mixture

The ethyl esters of linseed oil were prepared and fractionated so as to remove all saturated acids and oleic acid and some linoleic acid by the urea procedure used for linoleate. The iodine number of the preparation was 228, corresponding to a mixture of 24% ethyl linoleate and 76% linolenate.

ANALYTICAL PROCEDURES

The tissues of the chicks were separated as to carcasses and pooled organs (lungs, liver, heart, kidneys and spleen). The tissue was ground in a meat chopper and 50-gm samples extracted in a Waring Blendor for two minutes with 10 volumes of chloroform. This incomplete method of extraction was de-

[4] See footnote 2, page 326.

[5] The procedure for the fractionation of fatty acids or their esters by urea was obtained from Dr. Hermann Schlenk and Dr. Ralph Holman of this laboratory.

cided on since minimum manipulation rather than completeness of extraction is the required condition. The mixture was centrifuged and the chloroform layer dried over anhydrous sodium sulfate. The dried solution was filtered and evaporated to dryness by reduced pressure and a stream of nitrogen. The phospholipids were precipitated by acetone and $MgCl_2$, dissolved in moist ether, washed, dried over anhydrous sodium sulfate and evaporated to dryness by reduced pressure and a stream of nitrogen.

The acetone was evaporated from the soluble fraction by reduced pressure and nitrogen.

The polyunsaturated fatty acids were determined on the acetone soluble and insoluble fractions by the method of the American Oil Chemist Society ('49).

Since no standard exists for pentaenoic or hexaenoic acids, their quantities cannot be calculated but must be estimated on the basis of the $E_{1\,cm}^{1\%}$. Furthermore, their spectral effects on the less highly unsaturated acids cannot be determined, thus producing an error in the value given in tables 2, 3, 4 and 5. It was nevertheless decided to calculate the percentages of dienoic, trienoic and tetraenoic acids without the correction for the 5 and 6 double bond acids, since no idea of the amounts of these acids, especially the two and three double bond acids, can be obtained from extinction coefficients alone. For the purpose of these caculations it was assumed that all dienoic, trienoic and tetraenoic acids were linoleic, linolenic and arachidonic, respectively.

The cholesterol content of the acetone soluble fraction was determined by the method of Schoenheimer and Sperry ('35) in order to calculate the weight of triglyceride in the isomerized sample and the correct iodine number of the triglyceride fatty acids.

It was assumed that two-thirds of the phospholipids (acetone insoluble lipid) were fatty acid.

In order to determine the total amount of polyethenoid acid in chicks (table 6), complete extraction was necessary. An approximate 25-gm aliquot of the ground chick tissue was ex-

TABLE 2

Fatty acids in carcass neutral fat

RATION	DIENOIC ACIDS 233 mμ	TRIENOIC ACIDS 268 mμ	TETRAENOIC ACIDS 316 mμ	PENTAENOIC ACIDS 348 mμ	HEXAENOIC ACIDS 376 mμ
	%	%	%	$E^{1\%}_{1\ cm}$	$E^{1\%}_{1\ cm}$
Control	17.8	0.6	0.4	0.60	0.29
Fat-free [1]	1.3	0.3	0.0	0.00	0.00
Bayberry tallow [1]	1.1	0.2	0.0	0.00	0.00
Olein [2]	1.5	0.2	0.0	0.00	0.00
Wesson oil [1]	10.7	0.2	0.8	1.41	0.00
Linoleate [2]	6.6	0.1	0.4	0.72	0.00
24% Linoleate [2] 76% Linolenate	4.7	2.2	0.27	0.57	0.26
Cod liver oil [2]	1.6	0.0	0.7	0.76	0.17

[1] Average of two trials.
[2] One trial.

TABLE 3

Fatty acids in organ neutral fat

RATION	DIENOIC ACIDS 233 mμ	TRIENOIC ACIDS 268 mμ	TETRAENOIC ACIDS 316 mμ	PENTAENOIC ACIDS 348 mμ	HEXAENOIC ACIDS 376 mμ
	%	%	%	$E^{1\%}_{1\ cm}$	$E^{1\%}_{1\ cm}$
Control	16.3	0.8	0.9	3.26	1.58
Fat-free [1]	1.5	0.4	0.4	1.10	0.00
Bayberry tallow [1]	1.7	0.4	0.3	0.7 [3]	0.00
Olein [2]	0.9	0.5	0.0	0.00	0.00
Wesson oil [2]	11.0	1.2	2.3	2.80	0.00
Linoleate [1]	5.0	0.4 [3]	1.2	2.32	0.00
24% Linoleate [2] 76% Linolenate	4.8	0.9	2.4	5.10	1.99
Cod liver oil [2]	3.1	0.0	1.9	4.98	1.89

[1] Average of two trials.
[2] One trial.
[3] The value given is for one trial; the other showed no acid present.

TABLE 4

Carcass phospholipid fatty acids

RATION	DIENOIC ACIDS 233 mμ	TRIENOIC ACIDS 268 mμ	TETRAENOIC ACIDS 316 mμ	PENTAENOIC ACIDS 348 mμ	HEXAENOIC ACIDS 376 mμ
	%	%	%	$E^{1\%}_{1\ cm}$	$E^{1\%}_{1\ cm}$
Control	23.8	0.0	7.2	10.2	4.5
Fat-free [1]	7.3	1.0	3.6	0.0	0.0
Bayberry tallow [1]	6.0	2.6	1.0	0.0	0.0
Olein [2]	4.3	0.5	0.5	0.0	0.0
Wesson oil [2]	11.6	0.0	1.7	9.0	0.0
Linoleate [1]	8.8	1.1	3.3	8.3	0.0
24% Linoleate [2] 76% Linolenate	8.3	1.9	2.1	6.4	0.0
Cod liver oil [2]	10.8	0.0	4.0	6.0	0.8

[1] Average of two trials.
[2] One trial.

TABLE 5

Organ phospholipid fatty acids

RATION	DIENOIC ACIDS 233 mμ	TRIENOIC ACIDS 268 mμ	TETRAENOIC ACIDS 316 mμ	PENTAENOIC ACIDS 348 mμ	HEXAENOIC ACIDS 376 mμ
	%	%	%	$E^{1\%}_{1\ cm}$	$E^{1\%}_{1\ cm}$
Control	18.7	0.0	12.3	16.2	7.1
Fat-free [1]	6.2	10.2	2.5	3.5 [3]	0.0
Bayberry tallow [1]	5.9	6.7	2.1	1.8	0.0
Olein [2]	3.7	4.2	3.7	3.5	0.0
Wesson oil [2]	15.2	0.0	5.2	8.7	0.0
Linoleate [1]	10.1	6.7	6.4	6.3 [3]	2.4
24% Linoleate [2] 76% Linolenate	14.7	0.0	6.7	21.7	10.7
Cod liver oil [2]	11.0	0.0	6.3	19.4	9.5

[1] Average of two trials.
[2] One trial.
[3] The value given is for one trial; the other showed no acid present.

tracted three times by mixing with 125 ml of 3:1 alcohol-ether mixture in the Waring Blendor and heating to boiling. After each extraction the mixture was filtered with suction through a sintered glass filter. The residue was washed repeatedly with ether. The combined extracts were evaporated to dryness with reduced pressure and nitrogen, taken up in petroleum ether, dried and weighed. Samples of this were taken for fatty acid analyses. (The completeness of this procedure was tested

TABLE 6

The total amounts of polyunsaturated acids in 4-week-old chicks as compared with the amounts in an egg

CHICK NO.	RATION	DIENOIC ACID 233 mμ	TRIENOIC ACID 268 mμ	TETRAENOIC ACID 316 mμ	PENTAENOIC ACID 348 mμ	HEXAENOIC ACID 376 mμ
		gm	gm	gm	$E\,\frac{1\%}{1\ cm}$	$E\,\frac{1\%}{1\ cm}$
		From commercial hatcheries				
Egg	Stock	0.77	0.005	0.20	4.85	2.28
Chick [1]	Fat-free	0.30	0.05	0.07	Trace	0.00
		From hens on fat-free rations				
Egg	Fat-free	0.086	0.104	0.04	1.29	0.41
202	2% Wesson oil	6.470	0.284	1.825	0.0	0.0
204	Fat-free	0.084	0.034	0.036	0.0	0.5
205	Fat-free	0.204	0.089	0.0	0.0	0.0
3548	Fat-free	0.085	0.038	0.0	0.0	0.00

[1] These values were calculated from the analyses given in tables 2, 3, 4 and 5, the weights of the chicks and the lipid analyses of the carcasses and organs.

by comparing its results to those obtained with repeated extraction of small samples with alcohol-ether and ether, followed by regrinding and exhaustive ether extractions.)

RESULTS

The fatty acid contents of the tissue lipids are given in tables 2, 3, 4, and 5. Several general conclusions may be reached on the basis of these figures: (1) The same fatty acids are deposited in all the lipids and tissues, qualitatively and quantitatively, on the fat-free, bayberry tallow and olein ra-

tions; (2) organ lipids retain the higher polyunsaturated acids more tenaciously than the carcass lipids and the phospholipids more tenaciously than neutral fat; (3) diene acid is converted to tetraene, to pentaene, and possibly to pentaene acids; (4) triene acid is converted to all the polyethenoic acids studied; (5) after feeding cod liver oil there is a conversion of higher unsaturated acids to dienoic.

In table 6 are compared the total polyunsaturated fatty acid contents of chicks and those of eggs comparable to the ones from which the chicks were hatched. In the first part of the table the quantities of acids in eggs from hens on stock rations are compared to those in the 4-week-old chicks hatched from eggs laid by hens on stock rations and then fed the fat-free, bayberry or olein rations referred to in this paper (tables 2, 3, 4, and 5). The values for the acids in the chicks were calculated from the analyses given in tables 2, 3, 4, and 5, the lipid analyses of the carcasses and organs, and the weights of the carcasses and organs.

In the second part of the table are compared the quantities of acids in eggs laid by a group of 6 hens which had been on a fat-free ration for 15 weeks and in 4-week-old chicks hatched from similar eggs and fed the fat-free ration.

It may be seen that the chicks from stock eggs contained less acids than the eggs, the loss being especially marked with respect to the more highly unsaturated acids. (The trienoic acid values are probably antifacts due to the corrections required for their calculation.) The chicks from the hens on the fat-free ration showed no production of polyethenoic acids.

The values for the eggs are calculated from analyses of the pooled yolks of half the eggs laid by a group of 6 hens which had been on the fat-free ration for 15 weeks.

DISCUSSION

The explanation for the conversion in chick tissues of diene to tetraene and pentaene acids, or of triene to hexaene and others, is not obvious from our present knowledge of fatty acid

metabolism. The author has found that this also takes place in the fat of eggs (unpublished data). The reverse phenomenon, the conversion of the more highly unsaturated acids to dienoic, is easier to understand if one assumes the occurrence of simple hydrogenation. However, it may not be that simple, since the intermediate trienoic acid does not seem to be formed. Using hens on fat-free rations, the author has found (unpublished data) that the ingestion of trienoic acid will result in the deposition of trienoic in the first eggs, but in succeeding eggs the trienoic acid disappears, other polyethenoic acids replacing it.

The results strongly suggest that the chick cannot synthesize linoleic or linolenic acids. The work here reported shows certainly that the great bulk of polyunsaturated acids found in poultry come from the ration or the interconversion of polyunsaturated acids in the ration. The very small amounts in the tissues of chicks fed a fat-free ration are considerably smaller or no greater than in the eggs from which they were hatched, depending on whether the eggs were laid by hens on a stock or fat-free ration. In table 6 are shown the data from which this conclusion is drawn. The low polyethenoic acid content of 4-week-old chicks hatched from stock eggs and then placed on a fat-free ration approached that of chicks from eggs laid by hens on the fat-free ration. The latter were unable to increase the very low levels of the acids in the eggs from which they were hatched. The exception to this statement (chick 204) may have been due to a higher acid content of the particular egg from which the chick in question was hatched. The egg values given are of the pooled yolks of half the eggs laid by 6 hens on the fat-free ration.

Certainly, if the chick can synthesize linoleic or linolenic acids it must be in extremely small amounts. A study on the essential nature of these acids in the ration of growing chicks (Reiser, '50b) shows that they cannot be removed from the ration without deleterious effects.

CONCLUSIONS

1. On a fat-free ration the organ lipids of growing chicks retain the higher polyunsaturated fatty acids more tenaciously than the carcass lipids, and phospholipids more tenaciously than neutral fat.

2. Growing chicks convert dienoic acid to pentaenoic and possibly tetraenoic acids and convert trienoic acid to dienoic, tetraenoic, pentaenoic and hexaenoic.

3. The higher polyunsaturated fatty acids of cod liver oil are converted to dienoic acid.

4. Chicks cannot synthesize dienoic or trienoic acid, but produce their higher polyunsaturated acids from these.

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THE VITAMIN B_{12} CONTENT OF CHICK TISSUES AS INFLUENCED BY THE DIET [1,2,3]

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THREE FIGURES

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Since the isolation of vitamin B_{12} (Rickes et al., '48), the pure vitamin has been reported by several groups of workers to promote growth responses in chicks equivalent to those obtained with crude sources of the animal protein factor. Ott, Rickes and Wood ('48) stated that 6 μg of vitamin B_{12} per kilogram of diet stimulated chick growth as much as did crude sources of the factor. Nichol et al. ('49) reported that vitamin B_{12} could replace condensed fish solubles and injectable liver preparations in stimulating the growth of hyperthyroid chicks.

Evidence has also been accumulated to the effect that another factor or factors may be required in addition to vitamin B_{12} for maximum chick growth (Carlson et al., '49; Stokstad et al., '49; Combs et al., '50; Sunde et al., '50). In addition to these findings, Stokstad and Jukes ('50) reported that fermentation products of *Streptomyces aureofaciens* were found to promote growth in depleted chicks on diets containing ade-

[1] Supported in part by Project RG 1862, Division of Research Grants and Fellowships, U. S. Public Health Service, National Institute of Health, Bethesda, Maryland.

[2] The crystalline vitamin B_{12} used in the microbiological assays was generously supplied by Dr. D. F. Green, Veterinary Department, Merck and Company, Rahway, New Jersey.

[3] A preliminary report of this work was presented before the annual meeting of the American Institute of Nutrition, Atlantic City, New Jersey, April 19, 1950.

quate vitamin B_{12} and, further, that growth responses in chicks were obtained by feeding crystalline aureomycin hydrochloride. Lewis et al. ('49) reported the vitamin B_{12} content of various materials as obtained by the use of a rat growth assay technique.

The present study was initiated to study the effect of the diet on the vitamin B_{12} content of chick tissues.

EXPERIMENTAL

The New Hampshire chicks used in these studies were obtained from hens maintained on litter and which had been fed a ration containing animal protein (5% fish meal and 2% meat and bone scraps). The chicks were kept in batteries with raised screen floors, and feed and water were supplied ad libitum. The birds were weighed initially and at weekly intervals during an experimental period of 10 weeks.

The basal diet (G-1) used in this study was composed of 35% soybean oil meal, 60.75% ground yellow corn, 2% steamed bone meal, 1.5% oyster shell, 0.5% salt and 0.25% fortified fish oil (3,000 A–400 D). A riboflavin concentrate was used to supply approximately 2 mg of riboflavin per pound of diet. In diet G-2 the basal ration was supplemented with 5 gm of animal protein factor (APF) no. 1 (a charcoal adsorbate of vitamin B_{12})[4] per kilogram. The latter contained approximately 4 μg B_{12} per gram as determined by the microbiological assay described below. Ten per cent of dehydrated alfalfa leaf meal was substituted for an equivalent amount of ground yellow corn in diets G-3, G-4 and G-5, respectively. This sample of dehydrated alfalfa leaf meal had been shown to possess growth inhibitory qualities (German and Couch, '50). In addition, diet G-4 was supplemented with 5 gm of APF 1 and diet G-5 with 5 gm of APF 3[5] (standardized by the donor to contain 27.5 μg B_{12} per gram) per kilogram. Diet G-6 was the same as G-1 except that the former was supplemented with 2.5 gm

[4] Supplied by Merck and Company, Rahway, New Jersey.

[5] See footnote 4.

of APF 4 [6] per kilogram. Animal protein factor concentrate 4 contained approximately 0.6 μg per gram as determined by the microbiological assay described below.

Two experiments were conducted in the investigation presently reported. In the first test 125 chicks were randomized into 5 groups of 25 chicks each and were fed diets G-1 (basal unsupplemented), G-2 (5 gm APF 1 per kilogram), G-3 (10% alfalfa), G-4 (10% alfalfa and 5 gm APF 1 per kilogram) and G-5 (10% alfalfa and 5 gm APF 3 per kilogram), respectively. In the second experiment 75 chicks were randomized into three groups of 25 chicks each. The first group was fed the basal diet unsupplemented. Group 2 was fed the basal diet and in addition each chick received a weekly injection of 1 μg of vitamin B_{12} in 0.1 ml of water. The third group was fed the basal diet supplemented with 2.5 gm of APF 4 per kilogram.

At the end of the 10-week period three males and three females from each group were killed and the liver, kidney, pancreas and spleen were removed for analysis. The samples were each weighed and homogenized in a Waring Blendor with a measured quantity of water. An aliquot equivalent to 1 gm of tissue was pipetted into a 125-ml Erlenmeyer flask; 10 ml of pancreatin solution (5 mg/ml) were added and the volume was adjusted to 70 ml. The pH of the solution was adjusted to 7.0, the sample was overlaid with toluene, the flask was stoppered and the samples were incubated with shaking at 38°C. for 24 hours. The enzyme was destroyed by autoclaving for 5 minutes at 15 lb. pressure. After cooling, the samples were made up to 100 ml, filtered and stored at approximately 20°C. Dilutions for assay purposes were made from this stock solution. The method of Skeggs, Huff, Wright and Bosshardt ('48) was used to determine the vitamin B_{12} content of the tissues. Thiomalic acid was used in the medium as a reducing agent, as a growth factor for *L. leichmannii* and for the protection of vitamin B_{12}

[6] APF — control 8108–109 — was supplied by the Lederle Laboratories, Pearl River, New York.

during autoclaving. Assays were autoclaved for only 5 minutes prior to inoculation. Crystalline vitamin B_{12} was used as the standard in all assays. *L. leichmannii 4797* (American Type Culture Collection) was the test organism and acid production was the criterion used to measure the growth response.

RESULTS AND DISCUSSION

First experiment

It is apparent that when the all-vegetable protein diet (G-1) was supplemented with APF 1 (diet G-2), the growth rate of

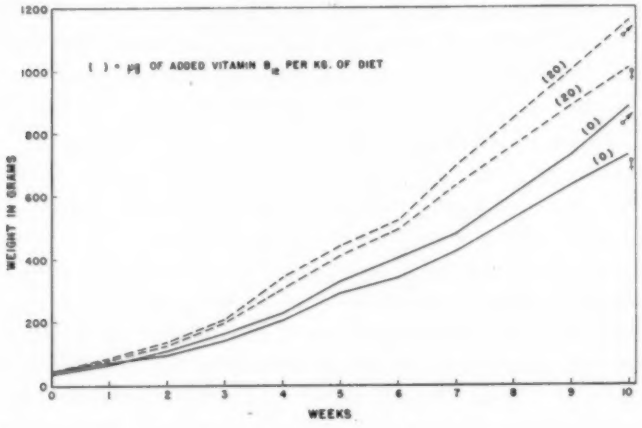

Fig. 1 Effect of APF concentrate no. 1 on the growth of New Hampshire chicks up to 10 weeks of age.

the chicks was improved (fig. 1). This is in agreement with the reports of Ott, Rickes and Wood ('48); Nichol et al. ('49) and Hill and Branion ('50). It is interesting to note that 10% dehydrated alfalfa leaf meal depressed the growth of the chicks in these tests (fig. 2) when compared to that of those not fed the inhibitory sample of alfalfa leaf meal (fig. 1). The addition of APF concentrate 1 increased the growth of the chicks fed 10% alfalfa (fig. 2). When the vitamin B_{12} content of the diet was increased approximately 7 times by adding

APF 3 (diet G-5), the growth of the birds was almost identical with that of those fed APF 1 (diet G-4), the growth curve of which is shown in figure 2. The fact that some samples of dehydrated alfalfa leaf meal do depress the growth of chicks has been reported by Lepkovsky et al. ('50), Peterson ('50) and German and Couch ('50).

It is quite apparent from table 1 that the vitamin B_{12} content of chick tissues is readily influenced by the supplementation of the all-vegetable protein diet (G-1) with an APF concen-

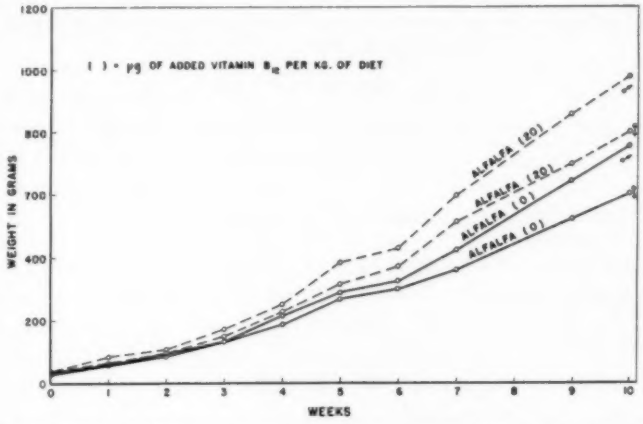

Fig. 2 Effect of APF concentrate no. 1 on the growth of New Hampshire chicks up to 10 weeks of age when fed with 10% dehydrated alfalfa leaf meal (inhibitory sample).

trate. The vitamin B_{12} content of the liver was increased approximately 5 times, that of the kidney approximately twice, and slight increases were noted in the B_{12} content of the pancreas and spleen as the result of feeding 5 gm APF concentrate 1 per kilogram of diet (calculated to supply 20 µg B_{12}/kg diet). It was pointed out above that 10% dehydrated alfalfa leaf meal depressed the growth of birds fed either with or without APF concentrate 1 (figs. 1 and 2). It is also apparent from table 1 that the B_{12} content of the liver, kidney, pancreas and spleen was decreased when the chicks were fed 10% de-

hydrated alfalfa leaf meal without vitamin B_{12} (comparison of diets G-1 and G-3, table 1).

Attention is directed to the fact that when APF concentrate 1 was fed with 10% dehydrated alfalfa leaf meal, the vitamin B_{12} content of the tissues was decreased in all cases except that of the kidney (diet G-4, table 1). It was mentioned earlier that when APF 3 was fed with 10% alfalfa (diet G-5), this supplementation failed to produce an increase in the growth

TABLE 1

Effect of feeding APF concentrates 1, 3 and 4 and injecting crystalline vitamin B_{12} on the vitamin B_{12} content of chick tissues[1]

(All values in μg/100 gm)

DIET	LIVER	KIDNEY	PANCREAS	SPLEEN
	First experiment			
G-1	3.8	4.6	5.7	10.8
G-2 (20 μg B_{12}/kg)	18.6	8.3	7.3	12.4
G-3 (10% alfalfa)	2.9	3.2	3.4	5.2
G-4 (10% alfalfa and 20 μg B_{12}/kg)	8.2	10.2	6.2	8.8
G-5 (10% alfalfa and 135 μg B_{12}/kg)	21.8	21.4	11.1	20.8
	Second experiment			
G1	4.1	3.9	2.5	5.4
G-1 (1 μg B_{12} injected per bird per week)	11.6	9.7	3.2	7.9
G-6 (2.5 gm APF 4/kg)	6.0	7.2	3.2	9.4

[1] All values are the averages of 6 determinations.

of the birds over that of those fed APF 1 with 10% dehydrated alfalfa leaf meal (diet G-4). However, the feeding of APF 3 (G-5) in this instance produced a two-fold increase in the vitamin B_{12} content of the tissues over that obtained when APF 1 was fed (diet G-4). It was pointed out earlier that APF 3 supplied approximately 7 times as much B_{12} in diet G-5 as did APF 1 in diet G-4. It is fully realized that crystalline B_{12} was not used in these tests. However, there is an apparent relationship between the vitamin B_{12} content of the diet and the

quantity of the vitamin stored in the liver, kidney, pancreas and spleen even when crude concentrates are used as sources of B_{12}. There is an indication that the B_{12} content of the tissues is influenced by the presence of 10% dehydrated alfalfa leaf meal in the diet when a suboptimum level of the vitamin is fed. When an excess of vitamin B_{12} is fed (diet G-5), this apparent effect on the storage of B_{12} in the tissues disappears, although the growth inhibition associated with this level of an inhibitory sample of alfalfa in the diet was not overcome by increasing the B_{12} supplementation approximately 7 times, through the use of APF 3. Attention is also directed to the fact that the liver appears to be the principal organ of storage of vitamin B_{12} in the chicken. This is in contrast to the rat, in which Lewis et al. ('49) reported that the kidney was the principal organ of storage for vitamin B_{12}.

Second experiment

The injection of vitamin B_{12} (1 μg per bird per week) produced an increase in the weights of both males and females at 10 weeks of age (fig. 3). It should be pointed out that the weights of the birds were further increased by feeding 2.5 gm APF 4 per kilogram of diet (fig. 3), although this level of APF 4 supplied only about 1.5 μg B_{12} per kilogram of diet. Stokstad et al. ('49) reported that fermentation products of *Streptomyces aureofaciens* contained an unidentified factor required for chick growth and Stokstad and Jukes ('50) reported that such fermentation products promoted the growth of depleted chicks fed diets containing an adequate amount of vitamin B_{12}. The aureomycin content of APF 4 was determined by the donor (Jukes, '50) and was reported to be approximately 4 mg of aureomycin per gram of APF 4. From these data it was calculated that 2.5 gm APF 4 supplied approximately 10 mg of aureomycin per kilogram of diet (G-6). Stokstad and Jukes ('50) have reported that growth responses in chicks have been obtained by feeding crystalline aureomycin hydrochloride. It is believed that the increased growth obtained by feeding APF

4 over that which resulted from injecting vitamin B_{12} might have been due to the aureomycin in the APF concentrate. It is apparent that the injection of vitamin B_{12} increased the content of the vitamin in the tissues (table 1). This increase in the B_{12} content of the tissues can be correlated with the corresponding increase in growth discussed above (fig. 3).

It is interesting to note that the vitamin B_{12} content of the liver and kidney from the birds fed APF 4 was lower than that of those which received diet G-1 plus an injection of 1 μg B_{12}

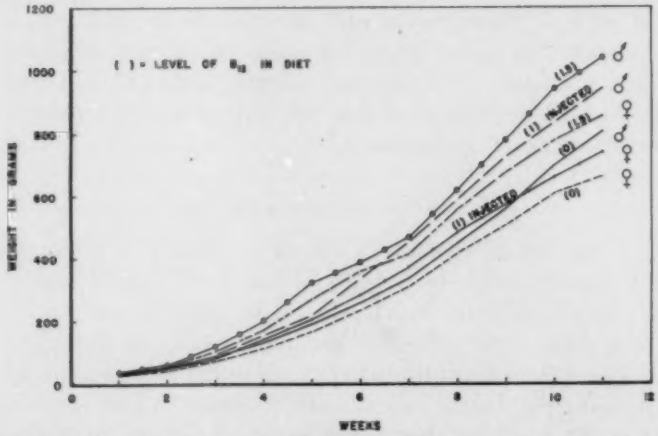

Fig. 3 Effect of vitamin B_{12} and APF concentrate no. 4 on the growth of New Hampshire chicks up to 10 weeks of age.

per bird per week. It should be pointed out that the level of B_{12} contributed by APF 4 (1.5 μg/kg) was rather low, and this may possibly account for the fact that the B_{12} content of tissues from chicks fed diet G-6 was lower than that of chicks injected with the crystalline vitamin (table 1). It was mentioned above that the growth of the birds fed APF 4 was greater than that of those injected with vitamin B_{12}. On the basis of the results of the second experiment it is believed that the vitamin B_{12} content of the liver and kidney is more closely correlated with the rate of growth than is the B_{12} content of the pancreas and

spleen (table 1). The latter was also apparent in the first experiment (table 1).

SUMMARY

The growth of New Hampshire chicks was increased by feeding an APF concentrate or by injecting crystalline vitamin B_{12}. The vitamin B_{12} content of the tissues has been shown to be related to the feeding of APF concentrates or to the injection of the crystalline vitamin, or both. The alfalfa sample used in this test depressed the growth of the birds and tended to decrease the vitamin B_{12} content of the tissues when groups fed equivalent levels of APF were compared. Such growth inhibition could not be overcome by feeding an excess of vitamin B_{12} in the form of an APF concentrate. APF concentrate 4 produced an increase in growth over that obtained by injecting crystalline B_{12}, and such an increase is thought to be due to the aureomycin contained in this concentrate. However, the B_{12} content of the liver and kidney from birds fed APF 4 was not as high as that of those from chicks injected with 1 μg B_{12} per chick per week.

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ASCORBIC ACID METABOLISM IN GUINEA PIGS IN RELATION TO GROWTH

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There is need for further information concerning the metabolism of ascorbic acid during rapid growth as compared to that in maturity, particularly with reference to possible differences in absorption, storage, utilization, and excretion of excess amounts. The present studies are concerned with these problems.

Dann and Cowgill ('35) reported that the dietary requirement for protection of guinea pigs against scurvy was approximately $\frac{1}{2}$ mg of vitamin C per 100 gm of body weight per day, irrespective of age. A possible discrepancy was apparent between these results and data reported from this laboratory (Reid, '48a) in which differences were found in the rate of utilization of the injected vitamin in animals at different ages. Approximately 23% of the dose (5 mg/100 gm body weight) was excreted in the urine by the full-grown animals and only about 11% by those in rapid growth. Later studies (Reid, '48b) with adult animals showed that probably a large portion of the vitamin, when administered in excess, is excreted into the gastrointestinal tract, where it is destroyed. An exact estimation of the amount thus disappearing could not be made, but it was found that as much as 15% of the total injected dose could be found at one time in the contents of the stomach and small intestine, with only small amounts in the contents of the cecum and large intestine. Since the contents of the upper part of the digestive tracts of these older animals are passed

fairly rapidly into the cecum and large intestine (Reid and White, '48), the results suggested that the vitamin is destroyed in the lower portion of the tract, particularly in the cecum. Conceivably, some destruction may also occur in the upper portion of the tract.

The experiments herein presented are concerned with the rates of accumulation of ascorbic acid in the blood, liver, kidneys, spleen, and adrenals of young animals growing rapidly, as compared to full-grown animals. An investigation has also been made of the excretion of excess amounts of the vitamin by the kidneys and gastrointestinal tract in the two groups of animals.

PROCEDURE

Two groups of inbred [1] guinea pigs, most of them males, one group averaging 71 days of age and weighing 467 gm and the other ranging in age from approximately one year to over three years, with an average age of 690 days and weight of 930 gm, were fed a commercial pelleted, vitamin C-free diet, supplemented with 1% of brewers' yeast, vitamin E, and a small amount of fresh carrots. Daily intraperitoneal injections of ascorbic acid (5 mg/100 gm in 0.5% $Na \cdot HCO_3$) [2] were given for at least three weeks before the studies were made. At periods varying from one-half hour to 24 hours after the usual daily injection, the animals were lightly etherized, blood samples taken from the hearts, and the pigs sacrificed. The liver, kidneys, spleen and adrenals were removed and extracted in 10% metaphosphoric acid and the extracts were frozen rapidly in solid carbon dioxide and kept in the deeply frozen state until the following day, when assays for ascorbic acid were made by both the indophenol and osazone (Roe and Kuether,

[1] The animals were from Family 13, a strain developed by the Bureau of Animal Industry (U. S. Department of Agriculture) and now being continued by the Genetics Unit of the National Cancer Institute, from which the animals used in the present test were obtained.

[2] A high dosage was necessary because the guinea pig does not excrete ascorbic acid in the urine unless the vitamin is administered in fairly large amounts (20 mg or more; Zilva, '36).

'43) methods. The contents of the stomach, small intestine, cecum and large intestine were also assayed by both methods.

RESULTS

The basal pre-injection levels of ascorbic acid (24 hours after the last injection) in the blood were 37% lower in the younger than in the older group of animals (table 1). Half an hour after administration of the vitamin more than a 7-fold increase was found in the blood in both groups. The content per unit volume of blood was higher in the older animals. By

TABLE 1

Average ascorbic acid in whole blood
(mg/100 ml ± S.D.M.)

HOURS AFTER LAST INJECTION	NO. OF ANIMALS	ANIMALS GROWING RAPIDLY	NO. OF ANIMALS	FULL-GROWN ANIMALS
0 (24)	7	0.51 ± 0.05	5	0.81 ± 0.08
½	6	3.79 ± 0.33	5	6.18 ± 0.27
1	6	3.47 ± 0.48	4	4.63 ± 0.58
2	5	2.87 ± 0.39	5	3.68 ± 0.19
3	6	2.51 ± 0.26	4	2.29 ± 0.28
4	4	2.27 ± 0.14	4	2.17 ± 0.10
6	9	1.60 ± 0.11	4	2.11 ± 0.15
8	4	1.43 ± 0.14	3	1.48 ± 0.16
10	4	1.36 ± 0.07	3	1.31 ± 0.18
24	7	0.51 ± 0.05	5	0.81 ± 0.08

the end of the first hour it had dropped 25% in the older animals and 8% in the younger group. Through the second and third hours the blood values decreased rapidly in both groups, though faster in the older animals, with the result that by the end of the 4th hour the levels in the two groups had become approximately the same. Between the 10th and 24th hours the loss from the rapidly growing animals must have been greater, since the 24-hour levels were significantly lower in this group, the difference between the two mean values being 5 times greater than the probable error of the difference.

The ascorbic acid concentration in the livers and kidneys showed some parallelism to the blood values. Lower pre-injection values for the vitamin were found in the livers of the younger animals (table 2). Following its administration, the average content in the livers of the growing animals rose 80% within half an hour, whereas the increase in the livers of the full-grown animals was only 44%. The maximum ascorbic acid values in terms of milligrams per cent did not differ significantly in the two groups but were reached earlier in the older animals with the higher basal level; after one hour as

TABLE 2

Average ascorbic acid in liver and kidneys
(mg/100 gm ± S.D.M.)

HOURS AFTER LAST INJECTION	LIVER		KIDNEYS	
	Animals growing rapidly	Full-grown animals	Animals growing rapidly	Full-grown animals
0 (24)	23.3 ± 2.6	30.8 ± 3.7	11.7 ± 1.0	12.9 ± 2.0
½	41.8 ± 1.8	44.3 ± 2.7	27.4 ± 2.2	32.6 ± 2.4
1	46.8 ± 3.4	55.2 ± 2.6	25.3 ± 2.5	31.1 ± 3.7
2	47.0 ± 1.2	53.3 ± 1.6	24.3 ± 1.4	22.3 ± 2.0
3	54.1 ± 4.9	50.3 ± 4.4	25.0 ± 1.3	20.5 ± 1.1
4	53.9 ± 3.1	50.0 ± 4.2	24.6 ± 1.8	16.8 ± 2.5
6	46.5 ± 2.8	49.4 ± 1.8	21.2 ± 0.8	16.7 ± 1.2
8	42.4 ± 3.1	51.4 ± 4.0	18.8 ± 0.8	15.3 ± 1.4
10	37.5 ± 0.6	43.2 ± 2.6	15.9 ± 0.9	12.1 ± 0.5
24	23.3 ± 2.6	30.8 ± 3.7	11.7 ± 1.0	12.9 ± 2.0

compared to after three hours in the younger group. On the basis of absolute amounts, the average maximum ascorbic acid values of the livers of the older animals rose 79% above the pre-injection levels, whereas the average maximum values of the livers of the younger group rose 132%. After the 4th hour the livers of the growing animals showed lower values at each successive period. The values for the older animals decreased more slowly.

The basal pre-injection values for the kidneys (table 2) were not significantly different in the two groups. Within half an hour after injection the content in both groups had more

than doubled. In the younger group it remained only slightly under this high level through the 4th hour, whereas by the 4th hour the maximum (one-half hour) kidney values for the older animals had dropped 48%.

The concentration of ascorbic acid in the spleen and adrenals showed no, or only a slight, relation to the concentration in the blood. In both the young and mature animals, the concentration values for the spleen showed no consistent variation with time of administration of the vitamin (table 3). The values for the younger group tended to be slightly higher, with an average of 75 mg% as compared to 67 mg% for the older animals.

TABLE 3

Ascorbic acid in spleen
(mg/100 gm ± S.D.M.)

HOURS AFTER LAST INJECTION	ANIMALS GROWING RAPIDLY	FULL-GROWN ANIMALS
0 (24)	68.0	66.5
½	75.6	71.3
1	77.8	64.2
2	76.6	69.1
3	68.6	70.8
4	76.7	64.8
6	80.1	62.8
8	76.4	66.6
10	74.1	62.5
24	68.0	66.5

The results obtained in the study of the adrenals are of special interest (table 4). In the older animals the ascorbic acid values, although fluctuating, bore little or no relation to the time of injection of the vitamin. There were marked variations in the size of the adrenals in this older group, which had a wide range of ages. In the young animals there was a progressive increase in the total ascorbic acid content up to the 10th hour and possibly longer. On the other hand, the reduced form, as determined by the indophenol method, remained fairly constant at a level somewhat lower than was obtained with the osazone method.

TABLE 4

Ascorbic acid in the adrenals
(mg/100 gm ± S.D.M.)

HOURS AFTER LAST INJECTION	ANIMALS GROWING RAPIDLY		FULL-GROWN ANIMALS	
	Reduced	Total	Reduced	Total
0 (24)	159.0 ± 12.1	169.8 ± 12.9	149.5 ± 8.8	158.3 ± 12.8
½	160.4 ± 4.1	169.1 ± 4.9	151.6 ± 11.9	176.5 ± 28.5
1	162.6 ± 9.2	179.4 ± 8.7	154.4 ± 10.3	195.1 ± 24.0
2	175.6 ± 1.5	185.4 ± 9.1	134.7 ± 11.1	146.6 ± 6.9
3	163.4 ± 7.6	189.4 ± 11.2	133.7 ± 12.7	160.3 ± 4.8
4	161.5 ± 6.2	196.9 ± 16.6	135.7 ± 7.1	132.8 ± 7.1
6	169.4 ± 5.2	197.2 ± 8.7	147.7 ± 4.8	144.1 ± 4.2
8	169.5 ± 3.9	204.8 ± 19.2	131.5 ± 1.6	141.0 ± 4.3
10	177.7 ± 6.6	228.1 ± 12.8	145.5 ± 9.5	152.6 ± 11.3
24	159.0 ± 12.1	169.8 ± 12.9	149.5 ± 8.8	158.3 ± 12.8

Table 5 shows the average rate of urinary excretion of ascorbic acid (μg/100 gm body weight/hour) and the percentage of the injected dose in the voided urine at successive periods as determined by the indophenol method. During the first hour the amount voided was found to be the same in the two groups, but during the second hour the mature animals

TABLE 5

Ascorbic acid voided in urine

HOURS AFTER INJECTION	μG/100 GM BODY WEIGHT/HOUR		PERCENTAGE OF INJECTED DOSE	
	Young [1]	Mature [2]	Young	Mature
0–1	55	56	1.10	1.12
1–2	41	168	0.82	3.36
2–3	138	329	2.76	6.58
3–4	92	231	1.84	4.62
4–6	24	162	0.96	6.48
6–8	24	17	0.96	0.68
8–24	8.6	1.2	2.75	0.39
	2.61 mg [3]	10.80 mg [3]	11.19 [3]	23.23 [3]

[1] Study made on 4 animals during a consecutive 5-day period. Each value in the table thus represents an average of 20 determinations.

[2] Study made on 10 animals during a two-day period, each value thus representing an average of 20 determinations.

[3] Total excreted per guinea pig in 24 hours.

voided the vitamin rapidly, whereas the younger group eliminated only one-eighth as much in terms of total amount per unit of body weight. This was a period of active storage in the liver, muscles, and doubtless in other tissues, their concentrations having been lowered considerably more in the young than in the mature animals in the 24-hour period since the last injection. From the second until the 6th hour the larger animals receiving the greater amounts of the vitamin voided more per unit of body weight, but from the 6th through the 24th hours the younger animals voided 3.6 times more. Osazone

TABLE 6

Ascorbic acid in contents of gastrointestinal tract

HOURS AFTER LAST INJECTION	TOTAL (MG)				MG/100 GM CONTENTS	
	No. of animals	Animals growing rapidly	No. of animals	Full-grown animals	Animals growing rapidly	Full-grown animals
0 (24)	4	1.3	7	2.5	2.6	3.8
½	4	2.8	4	3.4	5.4	5.5
1	4	2.4	4	3.7	4.5	5.5
2	5	3.7	8	4.5	7.4	7.9
3	4	3.6	4	5.3	6.4	9.8
4	3	2.4	6	7.7	4.6	10.9
6	5	2.4	6	5.7	4.5	7.8
8	4	1.8	3	3.5	3.9	5.2
10	4	2.1	3	3.1	4.5	4.1
24	4	1.3	6	2.5	2.6	3.8

tests made on some of the urines of the rapidly growing animals, collected from the 8th through the 24th hours, indicated that the substance titrated by the indophenol method was actually ascorbic acid. Irregularities in the completeness of emptying the bladder or in frequency of voiding in the young as compared to the old animals may have distorted the ascorbic acid values to some extent. However, since the figures presented each represent 20 test periods, these differences presumably would be almost, if not completely, equalized. Also, any such irregularities which may possibly have existed would not affect the 24-hour output figures.

The data obtained in the studies on the excretion of ascorbic acid into the gastrointestinal tract are summarized in table 6. The results expressed both as total amounts and as milligrams per cent showed lower values for the younger animals.

DISCUSSION

Ascorbic acid administered intraperitoneally is rapidly absorbed and circulated. However, it disappears from the blood more slowly than do many other metabolites. The basal (24-hour) values for the blood, livers and kidneys of the young animals in the present studies fell near the range of values found by Kuether, Telford and Roe ('44) for guinea pigs on a high dietary intake of the vitamin, but the values for the spleens and adrenals were considerably higher than they observed. The findings in the present studies of no difference in the basal values of the kidneys of young and mature animals and of lower basal levels in the livers of the young animals appear not to be in agreement with the observations of other investigators (Bessey and King, '33; Mulinos et al., '42; Torbina et al., '46; Kratinova and Bossis, '39a), who have reported higher values in younger animals. Bori ('47), on the other hand, found that the ascorbic acid content of the livers and spleens of guinea pigs did not change with age and that of the kidneys increased slightly. Kratinova and Bossis ('39b) reported that the ascorbic acid content of the kidneys increases up to the age of 18 to 24 months and then begins to decrease steadily. The variations in the findings with respect to guinea pigs among these different investigators may possibly be explained by differences in amounts of the vitamin given, in methods and time of administration, and possibly indirectly also by differences in other nutritive factors. In other types of rodents the effect of age on the ascorbic acid content of tissues is presumably also complicated by effects on the synthesis of the vitamin.

The present studies show that when ascorbic acid is given following a 24-hour period of deprivation, the livers of growing animals accumulate it more rapidly than do those of adults,

apparently not because of a capacity of the young livers to attain a greater saturation but because of greater depletion since the last injection.

The kidneys of the young animals tended to maintain a much more constant plateau between the first and 6th hours than did those of the mature animals. Whether these differences are related to utilization of the vitamin or to differing storage capacities or to differences in tubular reabsorption is not entirely clear. However, in view of the observations made previously (Reid, '48a) that a greater amount of ascorbic acid per unit of body weight disappears in the bodies of younger animals, it seems quite possible that these differences may, at least in part, be metabolic in origin.

There appears to be some mechanism operating to maintain approximate uniformity in concentration of the vitamin in both the spleen and adrenal regardless of wide fluctuations in concentration in the blood. No explanation or suggestion as to its nature can be advanced at this time. In view of the demonstrated importance of ascorbic acid in the metabolism of the adrenal cortex (Sayers et al., '44; Long and Fry, '45), the findings in the present study have considerable interest. In both the young and full-grown animals the reduced ascorbic acid in the adrenal did not appear to be influenced by administration of the vitamin. In older animals there was little variation in the total ascorbic acid, although some tendency for a saturation peak at one hour was noted. On the other hand, the young animals showed no peak corresponding to the blood curves but did show a progressive rise in the total ascorbic acid concentration up to at least the 10th hour, as measured by the osazone method. Whether this actually represented ascorbic acid as such, some derivative of ascorbic acid (Penney and Zilva, '45; Roe et al., '48), a conjugate of ascorbic acid (Lowenstein and Zwemer, '46), or some entirely different substance which will give this reaction is not apparent at the present time, although Vogt ('48) found no reaction in adrenal extracts containing a large fraction of the cortical steroids. Further studies, using methods for determining diketogulonic

acid as well as total and reduced ascorbic acid (Roe et al., '48), will be helpful. It is possible that the intraperitoneal injection of large doses of ascorbic acid at a fairly acid reaction, over a period of several weeks, may have produced adrenal stimulation which influenced the accumulation of ascorbic acid in this organ in the presence of large amounts of the vitamin available to it. Dugal and Thérein ('49) have shown that with continued stimulation the concentration of ascorbic acid in the adrenals rises.

Since the grinding and extraction of adrenal tissues can be effected quickly, it seems unlikely that appreciable oxidation occurred in the present experiments during these processes. Moreover, there is no apparent reason why more oxidation should occur in the third to 10th hour than in the first two hours, or why there should be a progressive increase in oxidation up to the 10th hour. It seems logical to suppose that, whatever the substances may have been which caused the observed increase in osazones in the third to 10th hour after administration of the vitamin to the growing animals, they were normal products of metabolism and not oxidation derivatives produced in the course of grinding and extraction.

Although lower ascorbic acid values were found in the contents of the digestive tracts of the younger animals, this does not necessarily indicate a significantly lowered excretion into the tract. Since the younger animals ate almost as much food as the older animals and the volume of the contents of their gastrointestinal tracts averaged 27% less than that of the older group, it follows that the food must have required less time to pass through the shorter tracts (small intestine particularly) of the younger animals (Eaton, '38). It would be expected, therefore, that the concentration of ascorbic acid in the contents of the digestive tracts would be lower at any given time, even though the total amount excreted into the tract daily might be equal in the two groups. Since no evidence was found of greater wastage of the vitamin in the gastrointestinal tracts of the younger animals, and since the kidneys of these animals excreted less of the vitamin per unit of body weight than did

those of the older pigs, it would follow that more may have been used endogenously within the body tissues of the younger animals.

SUMMARY

Studies have been made of the concentration of ascorbic acid in the blood and some of the internal organs of guinea pigs, following intraperitoneal injection of large doses of the vitamin. In the blood, liver, and kidneys changes in concentration were found at varying periods after injection, whereas the adrenals and spleen did not show this type of change. Differences were found between the young and mature animals with respect to the manner in which they handled the vitamin, the younger animals appearing to have considerably greater capacity for its conservation, in contrast to a seemingly greater wastefulness on the part of the older animals. The results suggest that more of the vitamin may have been used endogenously within the bodies of the young, rapidly growing animals.

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VITAMIN E DEFICIENCY IN CHICKS

I. THE EFFECTS OF DIETARY SUPPLEMENTS ON PLASMA TOCOPHEROL LEVELS AND VITAMIN E DEFICIENCY SYMPTOMS [1]

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FOUR FIGURES

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A positive correlation has been observed (Kinsey and Zacharias, '49) between the increased incidence of retrolental fibroplasia in premature babies weighing between 3 and 4 lb. at birth and the use of water-miscible vitamin preparations and iron in the care of these infants. Subsequently, Owens and Owens ('49) suggested that the disease is related to a deficiency of vitamin E in the diet of premature infants. They reasoned that the deficiency may be caused by low fat intake and absorption, and possibly aggravated by the use of large supplements of vitamin A and iron. The present investigation was undertaken to determine the effects of a water-miscible vitamin preparation, iron, and other substances on the occurrence of vitamin E deficiency symptoms and the plasma tocopherol levels of experimental animals, and to see whether vitamin E-deficient animals developed ocular abnormalities similar to retrolental fibroplasia.

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EXPERIMENTAL AND RESULTS

Male chicks (sex-link crosses of Rhode Island Red males and Barred Rock females) were used in these experiments because symptoms considered indicative of vitamin E deficiency are readily produced and recognized in chicks (Pappenheimer and Goettsch, '31; Dam, '44).

The chicks were raised in cages heated by light bulbs; 8 to 12 chicks were housed in each cage. The removable bottoms of the cages were cleaned and covered with fresh sawdust twice a week.

Food and water were given ad libitum. All diets were started when the chicks were one day old and were continued for the duration of the experiment (usually 6 or 7 weeks). The commercial chick diet used [3] is referred to, in this study, as the natural diet. The vitamin E-deficient basal diet, identical with Dam's diet 182 (Dam, '44) except for the salt mixture, contained:

Casein, alcohol extracted	15.0 gm
Dried yeast, Fleischmann type no. 2019	10.0 gm
Sucrose	54.6 gm
Gelatin	8.0 gm
Gum arabic	5.0 gm
Salt mixture 2 [4]	7.2 gm
l-Cystine	0.1 gm
Choline chloride	0.1 gm
Cod liver oil (mixed into the diet daily before feeding)	5.0 gm
A vitamin K substitute [5]	1.0 mg

All supplements were started when the chicks were two or three days old and were given orally by pipette, in the following quantities: synthetic dl-α-tocopherol [6] — 7 mg three times weekly; a water-miscible vitamin preparation [7] — 0.2 ml daily during the first week and 0.4 ml daily for the remainder of the

[3] Purina Mash Startena.

[4] General Biochemicals, Inc., Chagrin Falls, Ohio.

[5] Synkayvite, Hoffmann-LaRoche, Inc.,

[6] See footnote 4.

[7] Vi-Penta. Each 0.6 ml contains: 5,000 U.S.P. units vitamin A; 1,000 U.S.P. units vitamin D (activated ergosterol); 1.0 mg thiamine hydrochloride; 0.5 to 1.0 mg riboflavin; 50 mg ascorbic acid; 2 to 10 mg niacinamide.

experiment; ferrous sulfate [8] — 0.1 ml daily during the first week and 0.2 ml daily for the remainder of the experiment; ferric chloride [9] — 0.1 ml daily during the first week; the dose of ferric chloride was increased 0.1 ml each successive week for the remainder of the experiment.

The chicks were weighed twice a week. Each experiment included an appropriate control group.

Blood samples (0.2 ml) were obtained from each chick by heart puncture once a week. In experiments in which tocopherol supplements were administered, blood samples were taken approximately 24 hours after supplementation. The total tocopherols in the sample were determined by the micromethod of Quaife et al. ('49a), in which ferric ion is reduced to ferrous ion by the tocopherols present in the plasma sample. The ferrous ion reacts with α,α'-dipyridyl to give a red color. This color is measured with a Beckman spectrophotometer at 520 mμ. It is necessary to correct at 460 mμ for the presence of carotenoids, which also reduce ferric chloride and thus add to the color produced by tocopherol. Since xanthophyll is the chief carotenoid present in chick plasma, a correction factor was obtained for this substance. Crystalline mixed leaf xanthophyll [10] was used for this purpose. (The correction factor for xanthophyll was found to be 0.17, whereas for carotene it was 0.29.)

The experiments fell into 4 main groups: group I, chicks raised on the vitamin E-deficient basal diet; group II, chicks raised on the vitamin E-deficient basal diet supplemented with α-tocopherol; group III, chicks raised on the natural diet; group IV, chicks raised on the natural diet supplemented with α-tocopherol.

Weights

Chicks raised on the natural diet (with or without tocopherol supplementation) were the heaviest, those fed the basal

[8] Forty-two milligrams Feosol, ferrous sulfate in 1 ml water.
[9] Forty-one milligrams ferric chloride in 1 ml water.
[10] Kindly given by Professor George Wald, Harvard University.

diet were the lightest, and those fed the α-tocopherol-supplemented basal diet were intermediate in weight.

Occurrence of vitamin E deficiency symptoms

Group I. *Vitamin E-deficient basal diet.* A total of 84 chicks (10 experiments) were raised on the basal diet. Eighty-two of the 84 animals developed one or several of the symptoms of vitamin E deficiency, most frequently during the third week of the experiment. Histological sections of the eyes showed no abnormalities. Encephalomalacia (ataxia, tremors, leg spasms, stupor, and often death) as described by Pappenheimer and Goettsch ('31) developed in 54 animals. Histological sections of the brains of 17 of these animals revealed lesions identical with those described by Pappenheimer and Goettsch. Thirty of the 82 animals showed subcutaneous gelatinous exudates which were greenish in color. These exudates, according to Dam ('44), are symptomatic of vitamin E deficiency. In addition, gizzard necrosis, shown by Jungherr ('49) to be an accompaniment of field encephalomalacia in chicks, was found in 13 of the 82 affected animals.

Basal diet supplemented with the water-miscible multiple vitamin preparation. Nineteen chicks (two experiments) were raised on this diet. With the exception of two cases, in which atypical and delayed symptoms appeared on the 36th and 47th days, no symptoms of vitamin E deficiency were observed.

In order to determine the protective factor in the multi-vitamin preparation, its various constituents were used separately as supplements to the basal diet.[11] When oral supplements of the fat-soluble fraction of the multi-vitamin preparation were administered to 8 chicks fed the basal diet, symptoms of vitamen E deficiency developed in all of these birds. However, when the basal diet was supplemented with the water-soluble fraction, only one of 8 chicks developed symptoms. The vari-

[11] The fat-soluble and water-soluble fractions of Vi-Penta were kindly supplied by Hoffmann-LaRoche, Inc., Nutley, N. J. The vitamin content of these fractions was identical with that of whole Vi-Penta; each fraction was administered in the same quantity as whole Vi-Penta.

ous constituents of the water-soluble fraction were then tested separately.[12] From the results summarized in table 1, it may be seen that ascorbic acid is the protective substance in the multi-vitamin preparation.

Basal diet supplemented with ferrous sulfate. All chicks (7) in a group fed the basal diet and ferrous sulfate developed deficiency symptoms.

Basal diet without cod liver oil. (a) In 15 chicks two experiments) raised on the vitamin E-deficient basal diet from which the cod liver oil was omitted,[13] no symptoms developed during the 7 weeks of the experiment. (b) Identical results were obtained in another group of 16 chicks which were fed the vitamin E-deficient basal diet without cod liver oil for 4 weeks. At the end of this period, 5% cod liver oil was added to the diet. Eleven of the 16 chicks developed vitamin E deficiency symptoms, in most instances within a week after the addition of cod liver oil to the ration.

Group II. *Basal diet supplemented with α-tocopherol.* No symptoms of vitamin E deficiency appeared in 34 chicks (4 experiments) raised on the basal diet supplemented with α-tocopherol. Similarly, no deficiency symptoms occurred when chicks receiving the α-tocopherol-supplemented basal diet were given, in addition, supplements of the multi-vitamin preparation (22 animals) or ferrous sulfate (6 animals).

Groups III and IV. *Natural diet.* Twenty-three chicks raised on the natural diet showed no symptoms of vitamin E deficiency. The same results were obtained in separate experiments when the natural diet was supplemented with the following: the multi-vitamin preparation and ferrous sulfate (9

[12] Solutions of the various water-soluble vitamin constituents of Vi-Penta were prepared in the same concentrations as are found in whole Vi-Penta, and were administered to the animals in the same quantities.

[13] The diet of these chicks was supplemented with Aquasol (U. S. Vitamin Corporation, New York, N. Y.), an aqueous solution containing 1,000 units of natural vitamin A and 200 units of natural vitamin D per drop. The weekly dosage was: first and second weeks, one drop; third and 4th weeks, two drops; 5th week, three drops; remainder of experiment, 4 drops weekly.

TABLE 1

The frequency of appearance of vitamin E deficiency symptoms in birds fed the basal diet supplemented with various vitamins

DIET	NUMBER OF ANIMALS	NUMBER OF ANIMALS DEVELOPING VITAMIN E DEFICIENCY SYMPTOMS
Basal diet	11	9
Basal diet + thiamine	12	10
Basal diet + riboflavin	11	11
Basal diet + niacinamide	12	12
Basal diet + ascorbic acid [1]	12	3

[1] In two further experiments it was found that ascorbic acid prevented the occurrence of symptoms in only 12 of 23 chicks. In the 11 affected chicks, symptoms appeared approximately two weeks later than usual.

TABLE 2

The frequency of appearance of vitamin E deficiency symptoms in birds fed various rations and various dietary supplements

		DIET	PROPORTION OF ANIMALS SHOWING VITAMIN E DEFICIENCY SYMPTOMS
Group I	A.	Basal diet	82/84
	B.	Basal diet + Vi-Penta	2/19
	C.	Basal diet + Feosol	7/7
	D. (1)	Basal diet without cod liver oil + vitamins A and D (Aquasol)	0/15
	D. (2)	Basal diet without cod liver oil + vitamins A and D for 4 weeks; then changed to basal diet for three weeks	11/16
Group II	A.	Basal diet + α tocopherol	0/34
	B.	Basal diet + α-tocopherol + Vi-Penta	0/22
	C.	Basal diet + α-tocopherol + Feosol	0/6
Gorup III	A.	Natural diet	0/23
	B.	Natural diet + Vi-Penta + Feosol	0/9
	C.	Natural diet + 5% cod liver oil	0/11
	D.	Natural diet + 20% cod liver oil	11/17
Group IV	A.	Natural diet + α-tocopherol	0/29
	B.	Natural diet + α-tocopherol + 5% cod liver oil	0/12
	C.	Natural diet + α-tocopherol + crystalline vitamin A	0/19
	D.	Natural diet + α-tocopherol + ferric chloride	0/11

chicks), 5% cod liver oil (11 chicks), α-tocopherol (29 chicks), α-tocopherol and 5% cod liver oil (12 chicks), α-tocopherol and crystalline vitamin A (19 chicks), α-tocopherol and ferric chloride (11 chicks). The addition of 20% cod liver oil to the natural diet, however, resulted in the appearance of symptoms in 11 of 17 chicks.

The above results concerning the occurrence of vitamin E deficiency symptoms are summarized in tables 1 and 2.

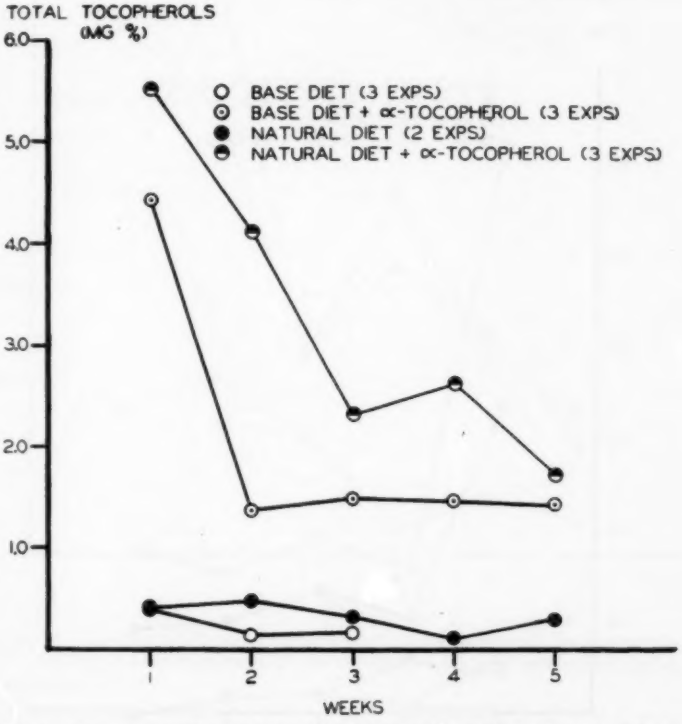

Fig. 1 The concentration of total tocopherols in the plasma of chicks raised on the 4 main diets. Each point represents the average concentration for an entire group. Chicks receiving the vitamin E-deficient basal diet (designated "base diet") could not be maintained after the third week. The commercial chick diet (Purina Mash Startena) is designed "natural diet."

Concentration of total tocopherols in the plasma

The plasma tocopherol levels of chicks raised on the various diets are shown in a series of graphs (figs. 1 to 4, inclusive). It may be seen from figure 1 that chicks raised on the vitamin E-deficient basal diet or the natural diet had average concentrations of tocopherol of approximately 0.2 mg% and 0.3 mg%, respectively. In chicks receiving either diet, these concentrations were not increased by the use of any dietary supplement except α-tocopherol.

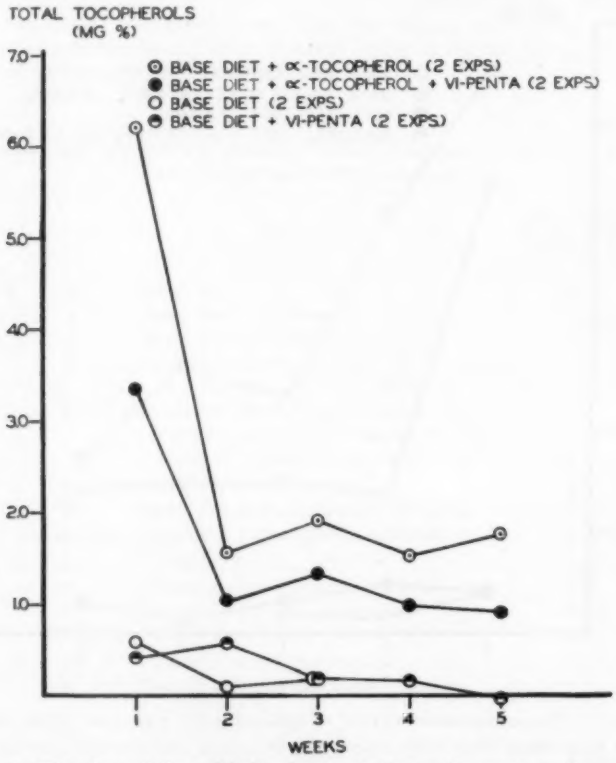

Fig. 2 The effect of the multi-vitamin preparation on the concentration of total tocopherols in the plasma of chicks raised on the vitamin E-deficient basal diet and on the basal diet supplemented with α-tocopherol.

Whereas the feeding of the multi-vitamin preparation to chicks raised on the α-tocopherol-supplemented basal diet lowered the tocopherol level (fig. 2), the administration of ferrous sulfate did not. Similarly, the addition of 5% cod liver oil or of

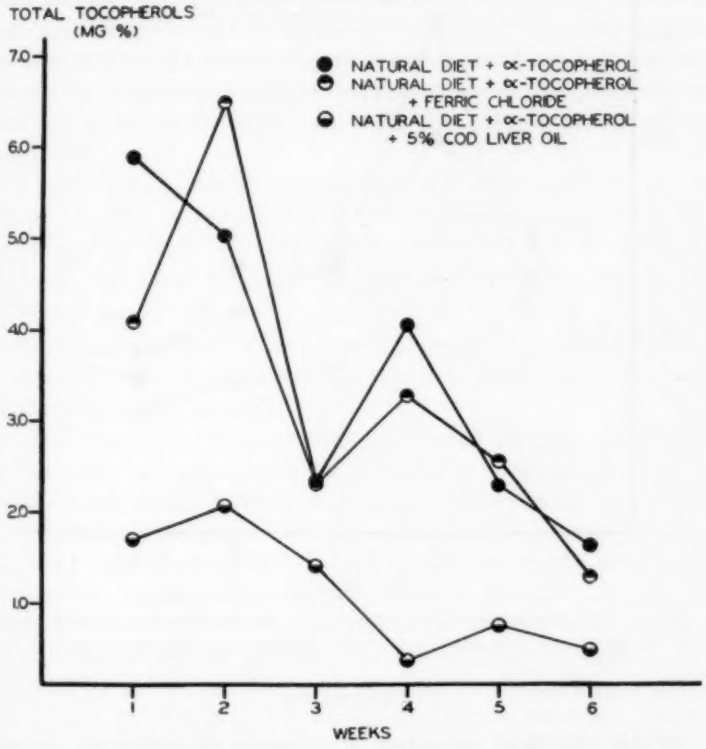

Fig. 3 The effect of 5% cod liver oil and of ferric chloride on the concentration of total tocopherols in the plasma of chicks raised on the α-tocopherol-supplemented natural diet.

crystalline vitamin A to the α-tocopherol-supplemented natural diet lowered the tocopherol concentration (figs. 3 and 4), while supplementation of this diet with ferric chloride (fig. 3), or with ferrous sulfate, had no effect.

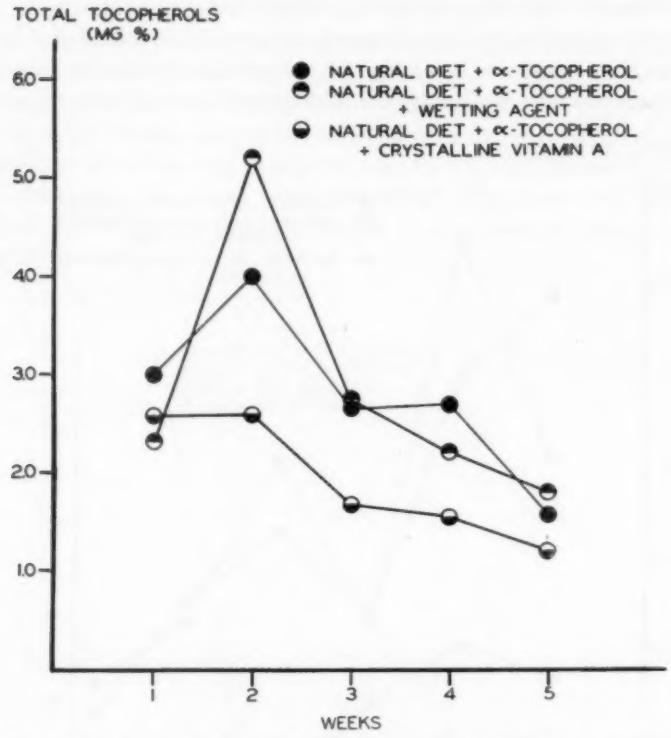

Fig. 4 The effect of crystalline vitamin A on the concentration of total tocopherols in the plasma of chicks raised on the α-tocopherol-supplemented natural diet.

DISCUSSION

While the chief symptom of vitamin E deficiency in the present experiments was encephalomalacia, cases of exudative diathesis and of gizzard necrosis were also observed. These results differ from those of Dam ('44), who observed exudative diathesis more often than encephalomalacia in chicks raised on his diet 182, and who did not observe gizzard necrosis. These differences may result from the use of a different salt mixture or a different strain of chicks.

Mason ('44), in paraphrasing Dam's conclusions concerning vitamin E deficiency in chicks, stated: "In short, unsaturated fatty acids in one form or another seem prerequisite to the appearance of exudative symptoms which, in turn, are considered cardinal signs of vitamin E deficiency in chicks and can be prevented by pure α-tocopherol." For this reason it was considered interesting to determine whether the occurrence of symptoms could be correlated with the presence of cod liver oil in the diet and with the plasma levels of tocopherol.

From table 3, which summarizes the data with respect to these factors, it may be seen that deficiency symptoms appeared only when cod liver oil was a constituent of the diet. Indeed, when cod liver oil was omitted from the vitamin E-deficient basal diet for a period of 4 weeks, no deficiency symptoms developed. When, however, at the end of this period, 5% cod liver oil was included in the diet, deficiency symptoms developed within a week.

With regard to the correlation between plasma tocopherol levels and the occurrence of deficiency symptoms, table 3 shows that chicks having high plasma tocopherol levels did not develop symptoms. On the other hand, it may be seen that in chicks showing low plasma tocopherol levels, deficiency symptoms developed in certain dietary groups and not in others. In several of the experiments the absence of symptoms in chicks having low plasma tocopherol levels is readily explained by the absence of cod liver oil in the diet (groups I D (1), III A, III B). There are instances, however, which cannot be explained on this basis. Chicks fed the basal diet supplemented with the multi-vitamin preparation (group I B) and those fed the natural diet supplemented with 5% cod liver oil (group III C) failed to develop symptoms, although their plasma tocopherol levels were as low as those of chicks receiving the vitamin E-deficient basal diet and cod liver oil was present in the ration. The absence of symptoms in these two groups indicates that there are substances other than vitamin

E, present in the natural diet [14] and in the vitamin preparation, which protect chicks from deficiency symptoms.

The protective substance in the multi-vitamin preparation has been shown to be ascorbic acid. This result is in accord with the findings of Dam et al. ('48), who observed that the addition of 0.5% ascorbic acid to a vitamin E-deficient basal diet protected chicks from deficiency symptoms. In addition, in an earlier communication Dam ('44) reported other substances (inositol and lipocaic) which protected chicks from vitamin E deficiency symptoms.

It would seem reasonable to regard the plasma tocopherol level as a reliable index of body stores of tocopherol, since both the body stores (Quaife et al., '49b) and the plasma tocopherol level [15] are directly related to the amount of α-tocopherol ingested. On this basis, ascorbic acid may be presumed to substitute for vitamin E (perhaps by virtue of its anti-oxidant activity), since chicks fed the multi-vitamin preparation along with the basal diet did not develop vitamin E deficiency symptoms despite low plasma tocophero levels (fig. 2). Evidence leading to a different interpretation was obtained by Dam et al. ('48), who found that in chicks fed their basal diet 97 supplemented with 0.5% ascorbic acid, the storage of tocopherol in adipose tissue was 110 μg/gm of fat, whereas the storage in chicks fed the unsupplemented basal diet was only 15 μg/gm of fat. Although no determinations of plasma tocopherol were given, their results seem to indicate that ascorbic acid does not substitute for tocopherol, but protects the tocopherol content of the tissue.

When either crystalline vitamin A or the multi-vitamin preparation was given to animals raised on diets which were supplemented with α-tocopherol, the plasma tocopherol levels

[14] The action of the protective factors in the natural diet can be overcome, in part, by feeding very large amounts of unsaturated fatty acids. For example, the addition of 20% cod liver oil to the natural diet was followed by the appearance of deficiency symptoms in 11 of 17 chicks. Information concerning one of the protective substances in the natural diet is given in the second paper of this series.

[15] Unpublished data from this laboratory.

were lowered.[16] It appears, therefore, that the vitamin preparation contains one factor (vitamin A) which lowers the plasma tocopherol level, as well as another factor (ascorbic acid) which protects the animals from vitamin E deficiency symptoms.

With regard to the administration of iron, the data showed that neither ferric nor ferrous salts, as administered, had any

[16] This is in accord with the observation of Mackenzie ('46) that the injection of pure vitamin A into rabbits increased the amount of tocopherol necessary to prevent creatinuria.

TABLE 3

Factors influencing the occurrence of vitamin E deficiency symptoms in chicks

		DIET	PLASMA-TOCOPHEROL LEVELS [1]	PRESENCE OF COD LIVER OIL IN DIET	OCCURRENCE OF SYMPTOMS
Group I	A.	Basal diet	low	+	+
	B.	Basal diet + Vi-Penta	low	+	—
	C.	Basal diet + Feosol	low	+	+
	D. (1)	Basal diet without cod liver oil + vitamins A and D (Aquasol)	low	—	—
	D. (2)	Basal diet without cod liver oil + vitamins A and D for 4 weeks; then changed to basal diet for three weeks	low	+	+
Group II	A.	Basal diet + α-tocopherol	high	+	—
	B.	Basal diet + α-tocopherol + Vi-Penta	intermediate	+	—
	C.	Basal diet + α-tocopherol + Feosol	high	+	—
Group III	A.	Natural diet	low	—	—
	B.	Natural diet + Vi-Penta + Feosol	low	—	—
	C.	Natural diet + 5% cod liver oil	low	+	—
	D.	Natural diet + 20% cod liver oil	low	+	+
Group IV	A.	Natural diet + α-tocopherol	high	—	—
	B.	Natural diet + α-tocopherol + 5% cod liver oil	intermediate	+	—
	C.	Natural diet + α-tocopherol + crystalline vitamin A	high	—	—
	D.	Natural diet + α-tocopherol + ferric chloride	high	—	—

[1] The designation "low" has been used for plasma tocopherol levels maintained after the first week at approximately 0.5 mg% or less; "intermediate" for levels of approximately 1.0 mg%; and "high" for levels of approximately 1.5 mg% or more.

effect on the tocopherol levels of chicks receiving the α-tocopherol-supplemented diets.

It seems unlikely that the use of water-miscible multiple vitamin preparations such as the one employed, and of iron, contributes to the production of a state of vitamin E deficiency in the organism. On the contrary, it appears that the use of a multi-vitamin preparation offers some protection against vitamin E deficiency in chicks.

SUMMARY

1. The present investigation was undertaken to determine the effects of a water-miscible multiple vitamin preparation, iron, and other substances on the occurrence of vitamin E deficiency symptoms and the plasma tocopherol levels of experimental animals, and to see whether vitamin E-deficient animals developed ocular abnormalities similar to retrolental fibroplasia.

2. The chief symptom of vitamin E deficiency observed in these experiments was encephalomalacia; cases of exudative diathesis and of gizzard necrosis were also observed. No ocular abnormalities were found.

3. Deficiency symptoms occurred only when cod liver oil was a constituent of the diet.

4. The plasma tocopherol levels of chicks fed the α-tocopherol-supplemented diets were lowered by the administration of a water-miscible multiple vitamin preparation, cod liver oil, or crystalline vitamin A. Iron supplements, in either the ferric or ferrous form, had no effect.

5. Chicks showing plasma tocopherol levels of approximately 1.0 mg% or more never developed symptoms. Chicks having plasma tocopherol levels of approximately 0.5 mg% or less developed symptoms only when fed certain diets. Chicks did not develop symptoms when fed the vitamin E-deficient basal diet supplemented with the multi-vitamin preparation, or when fed the natural diet supplemented with 5% cod liver oil. In both these groups, cod liver oil was present in the diet and the plasma tocopherol levels were as low as those of chicks

receiving the vitamin E-deficient basal diet. The absence of symptoms in these two groups indicates that there are substances other than vitamin E, present in the natural diet and in the multi-vitamin preparation, which protect chicks from vitamin E deficiency symptoms.

6. The protective factor in the multi-vitamin preparation was found to be ascorbic acid.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to Dr. Mary L. Quaife of Distillation Products Industries, Rochester, N. Y., for valuable instruction in the use of her micromethod for the assay of total tocopherols in blood plasma, and to Professor A. M. Pappenheimer of the Harvard Medical School, for his helpful suggestions and advice.

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AN UNKNOWN FACTOR WITH VITAMIN A ACTIVITY DISTILLED FROM LARD[1]

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TWO FIGURES

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We have previously reported ('50) on some nutritional aspects of the residue and distillate of molecularly distilled lard. In the present paper, we shall try to establish that there is present in lard a substance or substances different from the known forms of vitamin A but protecting animals against vitamin A deficiency.

MATERIALS AND METHODS

The experiments were carried out on a highly inbred colony of albino rats of the Sherman strain maintained for 15 generations on a purified diet similar to the one given in table 1. For this stock colony, crude casein or lactalbumin was used as the protein source, the lard residue was replaced by commercial lard, and percomorphum oil was added for vitamins A and D. The results as to growth and breeding (Kaunitz, '46; Kaunitz and Slanetz, '48) were satisfactory.

In a commercial laboratory[2] freshly rendered lard was subjected to molecular distillation at 215°C. and 10^{-3} mm Hg

[1] Aided by grants from the U. S. Public Health Service and the Williams-Waterman Fund of the Research Corporation.

[2] Distillation Products Industries.

pressure. Seven per cent was removed as strippings; this fraction will later be referred to as the distillate. The remaining material will hereafter be called the residue fraction. By this procedure the more volatile fractions, including vitamins A and E, were separated from the residue.

The distillate fraction was carefully studied for the presence of vitamin A and carotene. In the commercial laboratory the unsaponifiable fraction of the distillate was subjected to

TABLE 1

Composition of vitamin A-free diet

BASAL MIXTURE		SUPPLEMENTS PER KG [1]	
	%		
Lard residue	10	dl-Alpha-tocopherol acetate	25 mg
		Free dl-alpha-tocopherol	10 mg
Alcohol-extracted casein	30	Thiamine chloride	2 mg
		Riboflavin	4 mg
		Pyridoxine	4 mg
Cerelose	54	Nicotinic acid	100 mg
		Inositol	1,000 mg
Celluration	2	Choline	1,000 mg
		Vitamin K	4 mg
Salt mixture	4	*p*-Aminobenzoic acid	300 mg
		Calcium pantothenate	10 mg
		Calciferol	100 I.U.

[1] We are greatly indebted to Dr. Leo A. Pirk of Hoffmann-LaRoche, Inc., for generously supplying us with most of vitamins listed in tables 1 and 2. The Calciferol was kindly given to us by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

the antimony trichloride test. There was evidence of less than one unit of vitamin A per gram of non-saponifiable material.

We subjected to spectrophotometric analysis the unsaponifiable fraction of the distillate. Saponification had been carried out at 80°C. with a 6% alcoholic solution of KOH. Even assuming that the total absorption measured at 325 mμ was due to vitamin A, the maximum possible amount was less than 10^{-7} gm vitamin A per gram of distillate. Nor did the

antimony trichloride analysis of the distillate itself reveal any vitamin A.

Examinations of the iodine number, the thiocyanogen number, the melting point, and the peroxide number of both the distillate and residue fraction gave no significant differences. The distillate fraction contained 15 mg of tocopherols per 100 gm.

In table 1 the composition is given of the vitamin A-free diet used. The sources of protein and fat were extracted casein and lard residue. The diet was, therefore, for all practical purposes, free of vitamin A and carotene. Tocopherol was added as the synthetic dl acetate and as the

TABLE 2

Vitamin suspension for supplementary feeding

SUBSTANCE	MG PER ML OF WATER	SUBSTANCE	MG PER ML OF WATER
Thiamine chloride	4	Folic acid	5
Riboflavin	8	Biotin	0.05
Pyridoxine	8	Vitamin B_{12} [1]	0.5
Calcium pantothenate	20		

[1] Dr. Gladys Emerson of the Merck Institute for Therapeutic Research and Dr. Augustus Gibson of Merck and Company kindly supplied us with the vitamin B_{12}.

synthetic free form, the latter for its anti-oxidant action. The diet was made up at least once weekly and kept under refrigeration between feedings. In addition to the vitamins present in the ration, the rats received, twice weekly, one drop of the suspension given in table 2.

In order to compensate for any traces of vitamin A which may have been present in the diets with distillate supplements, and for purposes of assaying the distillate, control rats were supplied with graded doses of vitamin A. We decided to give the desired amounts by weekly subcutaneous injections rather than by feeding so as to be more accurate and to avoid possible intestinal destruction. A concentrated

solution of synthetic vitamin A palmitate [3] stabilized by Tween was diluted with saline solution immediately before the injection. Experiments were carried out to determine whether the material was properly absorbed. Figure 1 shows the influence of these graded doses on the survival rate after 37 days on the vitamin A-free diet. Two and one-half units gave a pronounced effect; the statistical probability, calculated by the Chi square method, was highly significant. In observations of the weight curves of 74 rats receiving graded doses of vitamin A palmitate it was noted that the rate of

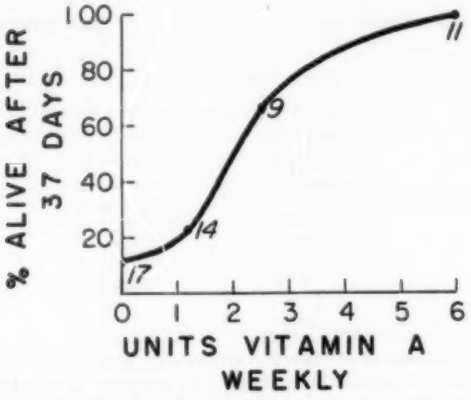

Fig. 1 Influence of graded doses of vitamin A palmitate on the survival rate of rats on a vitamin A-free diet. The numbers close to the points indicate the number of rats used.

[3] We are highly indebted to Dr. Leo A. Pirk of Hoffmann-La Roche, Inc., for supplying us with the synthetic vitamin A palmitate and advising us as to its use. With respect to some of the properties of the material, we owe the following information to Hoffmann-La Roche, Inc.: "Pure synthetic vitamin A and its esters were prepared by Isler, Huber, Ronco and Koffer (Helv. Chim. Acta., *30:* 1911, 1947) of F. Hoffmann-La Roche and Company, A. G., Basel, and shown to have physical constants agreeing with those in the literature for vitamin A from natural sources. Concentrates of acetate and palmitate, prepared according to the procedure of Isler et al., show the full activity in promoting growth and liver storage of vitamin A in rats expected on the basis of spectrophotometric assays. Pure, crystalline vitamin A acetate and palmitate obtained from these synthetic concentrates also show the same activity in rat growth and liver storage tests as crystalline material from natural sources."

growth was roughly proportional to the amount injected. It can therefore be concluded that the preparation used was properly absorbed and utilized.

The rats were placed on the experimental diet during the 4th week of life; paired litter mates of comparable weights and ages were selected so that the average weights of the various groups were the same. For reproduction experiments, the females were left with a male of proved fertility for a period of two weeks; thereafter they were examined for the "pregnancy sign" and weight changes for at least two weeks.

For the determination of fecal fat and food consumption, we followed essentially the technique of Hoagland and Snider ('42). Rats on a purified diet containing 30% lactalbumin, 10% lard residue, and 5,000 units of beta-carotene [4] per kilogram of diet were kept on wire screens in individual cages. Their litter-mate controls received commercial lard instead of the residue. Food consumption was determined daily and the feces were collected for one week. Then the rats were offered the same diet without fat. After two days, the procedure was repeated. The fat content of the feces on the fat-free diet was deducted from that on the fat-containing diet, and the resulting values were compared with the total fat intake. For the fecal fat determination, the powdered feces were treated with 5% trichloracetic acid, followed by the usual saponification and extraction. The differences between duplicate samples never exceeded 5% of the total fat content.

EXPERIMENTAL

In figure 2 are compared the average weights of animals receiving the distillate and those of their control groups on the vitamin A-free diet supplemented by weekly injections of 1.25 units of vitamin A palmitate. The weight curves were done according to the method of Zucker and Zucker

[4] Mr. R. C. Mosher of the Barnett Laboratories generously supplied us with crystalline beta-carotene and advised us as to some of its properties.

('42) by plotting the logarithm of the weight against the age on a reciprocal scale.

In figure 2 A are shown the results from two groups of 8 females each. To the diet of one 2% distillate was added. After 23 days on the diet the weight differences became sta-

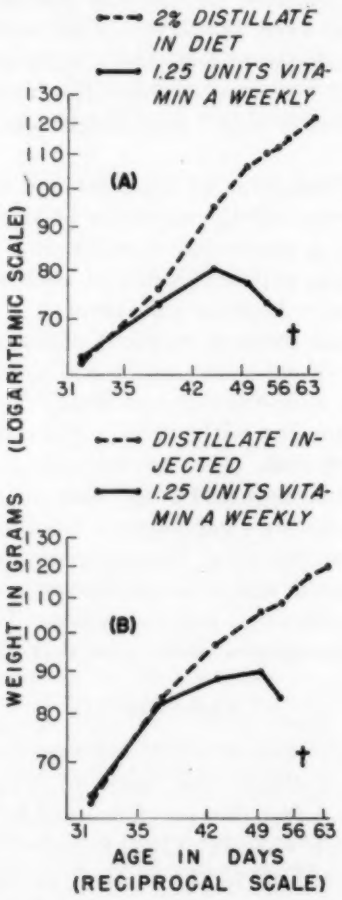

Fig. 2 Influence of feeding and injection of lard distillate on the weights of rats on a vitamin A-free diet.

tistically highly significant,[5] and the animals without distillate began developing severe eye changes. After 53 days on the diet, all but one of the distillate animals were alive and in good condition; in the control group, all had died. Similarly, in two groups of 12 females each, one of which was injected with 0.3 ml of distillate twice weekly, the average weight of the latter at 62 days was 139 gm, in contrast to 109 gm in the other group which had received three units of vitamin A palmitate by weekly injection. The "P" factor was 6.2.

For the experiments demonstrated in figure 2 B, the influence of distillate injection (0.3 ml twice weekly) was studied with 14 males and the same number of litter-mate controls. The latter received the same amount of lard residue by injection and 1.25 units of vitamin A palmitate weekly. After 26 days on the diet the weight differences became statistically significant, and the animals without distillate showed severe eye and fur changes. One week later, about 65% of the animals without distillate had died; the rest were in very poor condition and were killed for histological studies. All of the distillate animals were alive. After 8 weeks on the diet, the condition of the animals which had received 2% distillate in the diet was better than that of the animals injected with distillate. This seemed to be mainly due to the fact that the animals receiving the distillate injections finally developed abscesses at the site of some of the injections.

In table 3 are given the results of similar experiments carried out on 4 additional groups. In group 1, the weights of the distillate animals were significantly higher than those of the controls when the animals were 56 days old. When they were 67 days old, all controls had died but none of the

[5] For the statistical evaluation, the standard deviation was divided by the square root of the number of the experiments, giving the standard error of the mean of one series. The standard error of the difference of the means was found by taking the square root of the sum of the squares of the standard errors. If the difference between the means of two series, divided by the standard error of the difference of the means, gives a "P" factor of at least 2.0, a significant difference may be assumed.

distillate group, which were still growing after 8 weeks on the diet.

In groups 2, 3 and 4, the animals had been maintained on a diet containing crude lactalbumin and no free alpa-tocopherol but only alpha-tocopherol acetate until they were 56 days old. The distillate and vitamin A supplements indicated in the table were started at 42 days. At 56 days of

TABLE 3

Influence of lard distillate and various vitamin A palmitate supplements on the weights of rats on a vitamin A-free diet ("P" denotes the probability factor)

GROUP	NUMBER AND SEX OF RATS IN EACH GROUP	AGE	SUPPLEMENT	AVERAGE WEIGHT	P
				gm	
1	6♀	56	1.25 units vitamin A palmitate weekly	72	3.0
			2% distillate in diet	104	
2	7♀	84	1.25 units vitamin A palmitate weekly	91	7.8
			2% distillate in diet	155	
3	5♂	78	1.25 units vitamin A palmitate weekly	122	8.8
			2% distillate in diet	186	
4	8♀	107	7.50 units vitamin A palmitate weekly	112	3.0
			2% distillate in diet + 6 units vitamin A palmitate weekly	158	

age, the rats were placed on the vitamin A-free diet with extracted casein (table 1). When the animals in groups 2 and 3 were about 12 weeks old, the two groups without distillate showed severe deficiency symptoms, and several of them had died. The rest were sacrificed for histological studies. The distillate animals were alive and growing without deficiency signs when they were 16 weeks old.

In group 4, the diets offered and the timing of the supplements were identical with those of groups 2 and 3. The nature of the supplements is indicated in table 3. The animals receiving a weekly supplement of 7.5 units increased in weight until they were 11 weeks old and leveled off thereafter; the distillate group still grew normally at 16 weeks.

It is interesting to compare the distillate animals of groups 2 and 3 with the animals receiving 7.5 units of vitamin A and no distillate in group 4. The distillate animals, although they received no vitamin A supplements, were in much better condition than the group 4 animals when all were 16 weeks old. The distillate animals in all groups did much better as to weight and deficiency symptoms than two other groups not shown in the tables which had received 6 units of vitamin A palmitate weekly and no distillate.

In a further experiment, three groups of 9 males each received either no supplement, or three units of vitamin A palmitate by weekly injection, or 1% distillate added to the diet. At 9 weeks, the average weights were 96, 114 and 150 gm, respectively, with highly significant "P" factors.

In one set of experiments, 19 rats were kept on the deficient diet without supplement from weaning until they were 45 days old. At that time they had stopped growing and showed severe signs of A deficiency as evidenced by eye symptoms and roughened fur. Eleven of the 19 were then given 2% distillate in the diet; the remaining 8 received weekly 2.5 units of vitamin A palmitate, subcutaneously. After 8 days, the distillate animals had increased in weight from 75 to 85 gm; those on 2.5 units of vitamin A palmitate weighed, on the average, 79 gm initially and 77 gm after 8 days. Marked improvement of the deficiency symptoms was shown by 6 of the 11 distillate animals and by one of the controls. After 38 days on the distillate diet, when the animals were 83 days old, 5 were alive and growing well; all controls had died. It was obvious that distillate administration to animals with advanced deficiency signs had a decidedly curative effect in about half of the cases.

A variety of experiments were carried out on rats in which the experimental diet contained 30% lactalbumin instead of the extracted casein, 10% lard residue, and alpha-tocopherol acetate but no free alpha-tocopherol. To this diet had been added 5,000 units of crystalline beta-carotene per kilogram of ration. Most of the latter was rapidly destroyed, as chemical tests indicated, so that the rats received only varying, small amounts of the substance. This fact had to be taken into consideration in the preparation of the diets with distillate supplements. The distillate, containing an appreciable amount of free alpha-tocopherol, could have protected the carotene from destruction, so that differences between the two groups could have been explained merely as ordinary vitamin A deficiency. Therefore, the diets without distillate were fed one to three days after preparation, whereas the distillate was added to the distillate diets one week after the preparation of the ration.

Of 174 rats placed on this diet after weaning, about 75% developed various deficiency symptoms, especially reproductive failure in the female. Histological studies carried out by Dr. Herbert Stoerk [6] revealed the presence of lesions ordinarily associated with vitamin A deficiency, such as squamous cell metaplasia in various organs, keratitis, and kidney and bladder infections.

Twenty-five animals which were given weekly supplements of 2,000 units of crystalline beta-carotene by extra feeding showed no signs of pathological changes and, particularly, no reproductive failure. This indicates that the diet used was adequate in every respect except for its vitamin A content, and that the disease observed in the animals without additional carotene supplements was a true deficiency due to the absence of vitamin A or other factors capable of replacing vitamin A.

The occurrence of the disease could also be prevented by supplements to the diet of 1% of the lard distillate. In 43 animals thus treated, 50% of the litters were composed of

[6] Of the Merck Institute for Therapeutic Research.

normal young at weaning, in contrast to 10% in the unsupplemented group, the statistical evaluation showing high significance. In the unsupplemented group, nearly half of the animals died during the late stages of pregnancy; in the distillate group, over 90% survived pregnancy.

In the experiments with the carotene diet, an attempt was made to duplicate the protective action of the distillate by extra feeding of all known factors, including linoleic acid and cholesterol. These experiments, for which a total of over 100 rats were used, were negative except for alpha-tocopherol acetate, which exerted a beneficial effect in doses of 60 to 100 mg weekly. This effect, in the presence of a very low carotene intake, was probably due to the vitamin A-sparing action of tocopherols observed by Hickman and Harris ('46). Complete protection was obtained when 5% of dried brewers' yeast was added to the diet. The yeast, having been added during the preparation of the diet, probably protected the carotene from destruction by an unspecific anti-oxidant effect.

In order to rule out the possibility that the results obtained with animals on diets containing lard residue were due to the aversion of the rats to the diet, or to improper fat absorption, food intake and fecal fat excretion were determined. Paired littermates served as controls, receiving the same diet with commercial lard instead of the residue. In two groups of 24 animals each in which the food consumption was studied, the average daily intake was 10.8 gm for the animals on commercial lard and 11.1 gm for the residue group. Fecal fat excretion was studied on two groups of 8 animals each. Comparison of the fat intake with the excretion in the feces indicated that, in all instances, roughly 99% of the fat had been absorbed. Aversion to the diet and improper fat absorption were, therefore, not responsible for the occurrence of the disease; neither could toxic substances be responsible, because the addition of vitamin A or distillate prevented or cured the disease.

DISCUSSION

Molecular distillation of lard yields two fractions, the distillate of which possesses the capacity to counteract deficiency symptoms commonly considered the result of vitamin A deficiency.

Young animals receiving 2% of the distillate in the diet and consuming about 5 to 8 gm of food daily fared better than those receiving weekly supplements of 7.5 units of vitamin A palmitate. It is therefore certain that the vitamin A-like activity of 1 gm of the distillate exceeds that of 7.5 units of vitamin A palmitate. On the basis of analyses of the lard, the possibility can be ruled out that this vitamin A-like activity was due to the actual presence of vitamin A or carotene in the distillate.

In connection with the experiments in which carotene was added to the diet, we had thought of the possibility that the vitamin A-like action of the distillate might be caused by an unspecific anti-oxidant effect. The distillate, we thought, may have protected the traces of carotene left in the diet from destruction in the intestinal tract; however, the unequivocal outcome of the experiments in which the distillate was injected into animals on a vitamin A-free diet proved that the effect cannot be thus explained.

The most probable explanation so far possible is the presence in lard of a substance or substances chemically different from vitamin A but capable of replacing it. A very remote possibility is the presence of a substance capable of powerfully protecting any traces of vitamin A still present in the animals or in the food.[7] In particular the curative effect of the distillate on the depleted rats makes such an assumption highly improbable.

In this connection, the work of Dubouloz, Marville and Chevalier ('48) seems of interest. These workers observed that the chemical analysis of foodstuffs sometimes reveals the presence of less vitamin A than is accounted for by bio-

[7] We are grateful to Dr. Theodore F. Zucker of Columbia University for discussing this possibility with us.

assays. They came to the conclusion that there may exist still unknown forms or combinations of factors with vitamin A-like action. Various other observations are in accord with these findings, such as those of Mayer and Krehl ('48), who demonstrated that the isocaloric replacement of sucrose or corn oil by lard in vitamin A-deficient diets significantly improved the condition of the animals.

Considering that vitamin A is by no means plentiful in the natural rations of many animals, and that both carotene and vitamin A are easily destroyed by oxidation, the presence in animal fats of factors with vitamin A-like activity seems significant because the intake of such fats would protect the animals against deficiency.

These experiments strongly suggested that the various epithelial lesions commonly ascribed to vitamin A deficiency can be prevented or cured by a substance different from the known forms of vitamin A. These known forms are, therefore, not as essential for the maintainance of the normal epithelial functions as is generally assumed. This may also have some bearing on the validity of the results of some of the currently used bioassays for vitamin A.

Finally, the presence of this factor may be relevant to the question of a necessary minimum amount of fat in the diet. Animals can be maintained on a fat-free diet if all known nutrient factors, especially vitamin A, are generously supplied. If, however, as is often the case under natural conditions, the vitamin A level of the diet is low, the intake of animal fats may become essential.

SUMMARY

The nutritional effects on rats of the distillate and residue fractions of molecularly distilled lard were studied. When vitamin A-free diets containing the residue fraction as the fat source were supplemented by distillate, or when this material was injected, the animals could be protected against the signs of vitamin A deficiency. The protection given by

2% distillate in the diet seemed nearly complete and was much better than that provided by weekly injections of 7.5 units of vitamin A palmitate.

These results could be explained neither by the presence of vitamin A in the distillate nor by a nonspecific anti-oxidant action of the distillate. It was therefore concluded that lard contains a factor with vitamin A-like activity but which is chemically different from the known forms of vitamin A.

The significance of this factor with respect to specific epithelial lesions in animals on vitamin A-low rations, to vitamin A bioassays, and to a "fat minimum" in the diet has been discussed.

ACKNOWLEDGMENTS

We are deeply grateful to Dr. Philip L. Harris and the other members of the Biochemistry Department of Distillation Products Industries, a division of the Eastman Kodak Company, Rochester, N. Y., for their invaluable advice and criticism and for their assistance in supplying the processed lard as well as in conducting vitamin A and E analyses of the lard distillate.

To Dr. J. J. Beaver of Columbia University we are highly indebted for constructive criticism, particularly for help in the spectrophotometric analyses .

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VITAMIN E IN THE NUTRITION OF CATTLE

II. VITAMIN E LEVELS IN THE BLOOD, VITAMIN A AND CAROTENE UTILIZATION, AND OTHER CHEMICAL STUDIES [1]

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TWO FIGURES

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The possible importance of vitamin E in cattle nutrition has been discussed by Gullickson et al. ('49) in a recent paper, in which were also presented the results of experiments at this station having to do with the effect on the reproduction, health, milk production and growth of cattle of feeding rations low in vitamin E. In the present paper some further biochemical studies are reported, principally possible modifications in the chemical assay for tocopherols, estimations of blood plasma tocopherol levels, and the relations between a low vitamin E intake and the levels of vitamin A and carotene in the blood and liver.

METHODS AND RESULTS

Rations and test animals used

The preparation of the ration and the management of the animals are described in detail in the earlier report by Gullick-

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son et al. ('49).[2] Rat bioassays made on the ration whenever new lots of feedstuffs were used showed that the ration did not contain sufficient vitamin E to support reproduction in the rat (see also Gullickson et al., '49). In the present paper reference is made to 11 of the 30 animals used in the experiments over a 10-year period. Animals E-380, E-383, and E-390 were in the original group of 15 first put on test, and animals E-559 and E-573 were F_1 and F_2 offspring, respectively, of the original group of cattle. The vitamin A and carotene utilization experiments and most of the plasma tocopherol measurements were conducted with 6 of the last group of animals placed on experiment. Animals E-595, E-608 and E-612 received the unsupplemented ration, and the control group, E-607, E-611 and E-615, received in addition a supplement equivalent to 5 mg of tocopherol per kilogram body weight per day.[3]

Estimation of blood levels of vitamin E and of vitamin A and carotene

For the blood plasma tocopherol estimations reported in this paper, blood plasma samples were extracted essentially as outlined by Kimble ('39) and by Quaife and Harris ('44). For the colorimetric readings with the Emmerie-Engel ('38)

[2] The animals were fed rice straw ad libitum and a concentrate mixture of the following composition:

Polished rice	25%
Brewers' dried grains	30%
Solvent-extracted distillers grains	18%
Corn starch	11%
Dry skim milk	9%
Steamed bone meal	2%
Iodized salt	1%
Rendered lard	4%
Delsterol (2,000 D)	0.6 gm per lb.

Unless otherwise noted all animals received 10,000 I.U. of vitamin A daily per 100 lb. of body weight.

[3] Fed as a powdered concentrate containing 50 or 100 mg of mixed tocopherols per gram, furnished through the courtesy of Dr. P. L. Harris, Distillation Products, Inc., Rochester, N. Y. According to the manufacturer, the mixed tocopherols had 75 to 90% of the biological activity of d,l-α-tocopherol.

method, the residue from extraction of 5 to 10 ml of blood plasma was dissolved in 3.0 ml of petroleum ether and 1.0 ml of absolute ethanol, and 0.5 ml of the ferric chloride-2,2′-bipyridine reagent in glacial acetic acid (Devlin and Mattill, '42) was added. The optical densities were read after 4 to 6 minutes at 510 mμ against a suitable blank in a Coleman Junior spectrophotometer adapted for use with 3.0- to 4.5-ml volumes in 19-mm tubes. An internal α-tocopherol standard (Kaunitz and Beaver, '44) was used as the basis for calculations of the approximate tocopherol content because of the inhibition of color development in the plasma extract. The magnitude of this inhibition increased with an increase in the amount of added d,l-α-tocopherol, as is shown in table 1. The experimental animals had low blood plasma carotene levels during most of the experiment, hence the corrections for the carotene interference were small. However, because of the aforementioned and other known limitations of the assay procedure (Baxter, '47), the values herein are reported in terms of total fat-soluble reducing substances calculated as α-tocopherol.

TABLE 1

The inhibition of color development by blood plasma extract

(The determinations were made as outlined in the text. The d,l-α-tocopherol was added to the extracts representing 5 ml of a composite blood plasma sample just prior to the final color development.)

ADDED D,L-α-TOCOPHEROL	OPTICAL DENSITY AT 510mμ		INCREASE IN OPTICAL DENSITY PER 15 μG INCREMENT OF ADDED TOCOPHEROL	
	Without plasma extract	With plasma extract	Without plasma extract	With plasma extract
μg				
0	0	0.108	....	....
15	0.155	0.253	0.155	0.145
30	0.297	0.381	0.142	0.128
45	0.440	0.494	0.143	0.113

Plasma vitamin A and carotene determinations were made on 5.0- or 10.0-ml samples, essentially as described by Kimble ('39) but with mild saponification, as suggested by Bessey et al. ('46), to insure complete extraction of vitamin A.

Estimations of vitamin A and carotene in liver samples

The analyses were made on 20- to 120-mg liver samples removed by a simple biopsy procedure.[4] The samples immediately after removal were freed of excess blood by being blotted on filter paper and were then dropped into weighed test tubes containing a measured volume (2 to 3 ml) of absolute ethanol and 40 μg of α-tocopherol to serve as an antioxidant. Subsequently the tubes were reweighed, the samples homogenized in a glass homogenizer (Potter, '49) and then aliquots removed and heated with an equal volume of 2 N aqueous KOH for 30 minutes at 60°C. After cooling, the samples were extracted with petroleum ether and the extracts analyzed as with the blood plasma assays.

The disadvantage of using small samples which gave relatively low final optical density readings was offset by the possibility of obtaining samples at intervals from the same animal. Reasonable agreement in values was obtained for two separate samples taken at the same time from each of 6 animals; the values showed an average difference of 11% (range 5% to 24%). These results and the report of With ('46) that the vitamin A content of various portions of liver is relatively constant make valid the use of a small biopsy sample in estimating the storage in the liver as a whole.

Experiments on procedures for eliminating interference in the tocopherol assay

The interference due to carotene may be corrected for if the carotene values are low, or largely eliminated by the selective hydrogenation procedure of Quaife and Biehler ('45). The

[4] The liver samples were obtained through the cooperation of Dr. A. J. Sellers and Dr. Donald Jasper of the Division of Veterinary Medicine.

simple alternate procedure reported here is based on the oxidation of α-tocopherol to the α-tocopherylquinone under conditions where carotene is changed to colorless products which show no light absorption above 260 mμ and which do not react with the ferric chloride-2,2′-bipyridine reagent following reductive cyclization of the α-tocopherylquinone to α-tocopherol (Tishler and Wendler, '41).

TABLE 2

Removal of carotene interference by the oxidation-cyclization procedure

(The amounts of tocopherol and carotene are those present in the initial 10-ml volume used for the procedure described in the text. One-milliliter aliquots of the final petroleum ether extract were used for the color development.)

AMOUNT OF TOCOPHEROL	AMOUNT OF CAROTENE	OPTICAL DENSITY AT 510 mμ		RECOVERY OF TOCOPHEROL WITH CAROTENE DESTRUCTION
		Without carotene destruction	With carotene destruction	
μg	μg			%
692	0	0.679	0.688	101
692	27	0.713	0.683	101
692	108	0.762	0.672	99
0	108	0.055	0.010	..
508	0	0.525	0.520	99
508	254	0.90	0.506	96
508	508	1.0	0.502	95
..	508	0.8–0.9	0.008	..

Samples containing 0.1 to 2 mg of tocopherol and up to 0.5 mg of carotene in 5 to 10 ml of ethanol are treated with 2.5 to 3.0 ml each of 0.2% $FeCl_3 \cdot 6H_2O$ and 0.5% 2,2′-bipyridine in ethanol and then heated in a water bath at 65° to 70°C. for 50 minutes, with addition of ethanol to replace that lost by volatilization. Two to 3 ml of 0.5 M $SnCl_2$ in concentrated HCl are added, and the tubes reheated at 75°–80°C. for 45 minutes, with lost alcohol being replaced. After cooling, the samples are extracted with 10.0 ml of petroleum ether or isooctane, the

extracts washed twice with water and 1.0- to 3.0-ml aliquots pipetted into colorimeter tubes for assay of tocopherol using the acetic acid reagent. As is shown by the data presented in table 2, the procedure gave satisfactory results even with carotene to α-tocopherol ratios of one or greater, which are larger than those usually encountered in blood samples. In limited trials with blood plasma extracts, full recovery of added tocopherol within a reasonable range of experimental error was obtained.

Attempts to simplify the Scudi and Buhs ('42) procedure for routine estimation of blood tocopherol with use of sodium hydrosulfite as a reducing agent were unsuccessful. Although the requisite conversions and extractions could readily be carried out if exposure to alkali were omitted, the addition of strong alkali required for separaton of α-tocopherylhydroquinone from other fat-soluble substances resulted in erratic losses even under carefully controlled conditions and in the presence of excess hydrosulfite.

Blood plasma fat-soluble reducing substances

Assays of blood plasma samples from animals E-559 and E-573, taken after the animals had been on the unsupplemented ration for about 28 and 26 months, respectively, gave values for total fat-soluble reducing substances, calculated as α-tocopherol, of less than 100 μg%. Animal E-573 died suddenly 20 days later, two days after calving.[5] Animal E-559, which showed definite cardiac abnormalities similar to those reported previously (Gullickson and Calverley, '46), was then given a supplement of 2 gm of mixed tocopherols daily. After two weeks the calculated tocopherol level was 360 μg% and the average value of 5 later estimations over a 9-month period was 770 μg%. Three weeks after supplementation was begun, animal E-559 calved normally and remained ap-

[5] A total of 13 unsupplemented animals showed similar sudden deaths during the course of the experiments, the tendency for which was accentuated by pregnancy and calving (Gullickson et al., '49).

parently healthy until removed from experiment 18 months later.

Estimations of blood plasma tocopherol levels were also made on a later group of three supplemented and three unsupplemented animals during a period approximately one and one-half to two and one-half years after the animals had been placed on experiment. The results are summarized in table 3. The vitamin A and carotene levels in the blood samples used were under 30 and 150 μg%, respectively, and thus corrections made for their presence were relatively small. Although the

TABLE 3

Total fat-soluble reducing substances in the blood plasma of experimental animals

DATE	TOTAL FAT-SOLUBLE REDUCING SUBSTANCES EXPRESSED AS MICROGRAMS α-TOCOPHEROL PER 100 ML					
	Unsupplemented animals			Supplemented animals		
	E-595	E-608	E-612	E-607	E-611	E-615
12– 4–47	160	174	242	501	546	677
3–12–48	161	178	299	1020	845	1120
7–13–48	470	239	296	557	552	703
11– 3–48	316		273	748		614
1–27–49	260		120	555		468
	Mean = 249 ± 16.3 [1]			Mean = 685 ± 37.7 [1]		

[1] Probable error of mean.

supplemented animals were fed mixed tocopherols, it is probable that the tocopherol in the blood was predominantly α-tocopherol, inasmuch as Quaife and Dju ('49) have shown that the livers of cattle fed normal rations contain no γ or δ-tocopherol. The calculated blood plasma tocopherol values given in table 3 may be compared with the values of 685 and 582 μg% reported for cows on pasture, and on grass, hay and silage, respectively (Whiting et al., '49) and with the values of 724 to 1473 μg% for cows on pasture and 198 to 450 μg% for barn-fed cows approximately 35 days prepartum reported by Latschar et al. ('49). The lower values observed earlier for

animals E-559 and E-573 may have resulted from these animals' being F_1 and F_2 generation animals, respectively; from a possible lower vitamin E content of the ration at this earlier period; or from the decrease in serum tocopherol levels reported to occur shortly before parturition (Latschar et al., '49).

Relation between low tocopherol intake and utilization of vitamin A and carotene

A study of this relation was of particular interest because of the favorable effect of vitamin E on the utilization of vitamin A and carotene by laboratory animals (Moore, '45) and the importance of vitamin A and carotene in cattle nutrition. The possible effect on the utilization of vitamin A and carotene of feeding a low vitamin E ration to cattle was measured by estimating the blood and liver levels of vitamin A and carotene in the two groups of animals listed in table 3 during periods when the animals received controlled intakes of vitamin A or carotene. Carotene supplementation in both groups was started at two to 8 weeks after the animals had been placed on the experiment, and was continued for one year. Thereafter, for both groups of animals, the carotene was replaced by vitamin A for a period of two months. The carotene (in oil)[6] and vitamin A (as fish liver oil) supplements were given daily by capsule.

The results of this experiment presented in figures 1 and 2 show that the blood plasma vitamin A and carotene levels of the two groups on the initial carotene intake of 75 μg per kilogram of body weight per day and with the later increased intakes of 100 and 300 μg per kilogram were approximately the same. Similarly, the increase in blood plasma vitamin A, the decrease in blood plasma carotene, and the liver storage of vitamin A following vitamin A supplementation were essentially the same for both groups. The similar responses of the

[6] General Biochemical Company, Type 300, containing 50,000 U.S.P. units per gram.

unsupplemented and supplemented animals warrant the conclusion that feeding the low vitamin E ration did not adversely affect the utilization of carotene or vitamin A as measured by blood plasma levels and liver storage. Although these animals were probably less deficient in tocopherol than were the earlier animals on experiment, as evidenced by the cal-

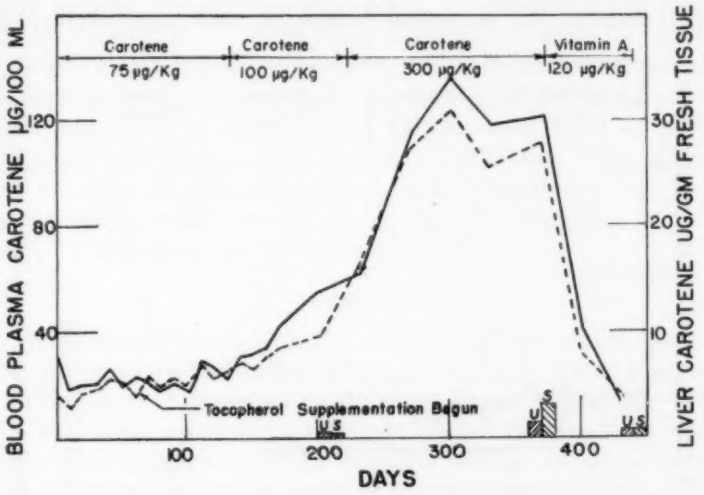

Fig. 1 Blood plasma and liver carotene levels of cows on a vitamin E-deficient ration, with and without tocopherol supplementation. The blood plasma level for the unsupplemented animals is represented by the solid line, and that for the supplemented group by the broken line. The bars at the foot of the graph show the results of the liver assay, those marked U representing the unsupplemented, and those marked S the supplemented, animals.

culated plasma tocopherol levels and by the lack of sudden deaths in the unsupplemented group, nonetheless the fact that the ration used was low in tocopherols was demonstrated by the relatively low tocopherol levels of the unsupplemented group and the failure of the ration to support reproduction in the rat.

Miscellaneous blood, urine and tissue analyses

Monthly determinations of the blood plasma ascorbic acid and calcium and phosphorus levels of the unsupplemented animals over a two-year period in the early part of the experiment showed that these constituents were present in normal concentrations. At different times during the experiment the blood cholesterol and hemoglobin, and the urinary creatine to creatinine ratios of the unsupplemented

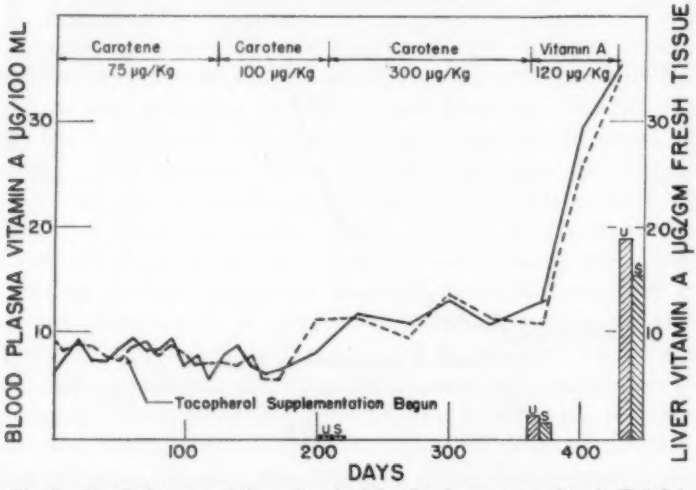

Fig. 2 Blood plasma and liver vitamin A levels of cows on a vitamin E-deficient ration, with and without tocopherol supplementation. The lines and bars have the same significance as those in figure 1.

animals, were determined and these likewise fell within the normal range. Analyses for riboflavin, nicotinic acid and choline in the liver of one animal which had died suddenly while on experiment indicated that these substances were present at normal levels.

Relation of the low vitamin E intake to milk composition

Analyses of the milks from animals E-380, E-383 and E-390, which were reared on the deficient ration, showed that

the composition of the milks was within the normal range for specific gravity, organic matter, ash, casein, albumin, lactose, ascorbic acid, thiamine, riboflavin, nicotinic acid and vitamin A. The reduced vitamin E intake was reflected in a deleterious effect on the keeping quality of the butterfat, as shown by accelerated oxidation tests (Burr, Lundberg and Chipault, '46). The fat level in the milk was somewhat lower than normal. In later experiments a decreased level of milk fat was noted in the milks from the group of animals which received tocopherol supplements as well as in the milk from the unsupplemented group. At the termination of the experiment both groups of animals showed an increased level of milk fat when placed on normal rations (Gullickson, '50). Thus the low level of milk fat noted when the animals were on the experimental ration was probably due to properties of the ration other than the tocopherol content.

SUMMARY

Estimations have been made of the total fat-soluble reducing substances, calculated as α-tocopherol, in the blood plasma of dairy cattle fed rations in which the amount of vitamin E was below that measurable by rat bioassay. Two animals, one which died 20 days later and one with cardiac abnormalities, had calculated tocopherol levels of less than 100 μg %. Similar measurements for a later group of three unsupplemented animals over a 14-month period gave an average value of 249 μg %, considerably lower than the value of 685 μg % found for a corresponding group of animals supplemented with mixed tocopherols, and also lower than values reported in the literature for animals fed normal rations.

Measurements of the blood plasma carotene and vitamin A levels of three supplemented and three unsupplemented animals on controlled carotene and vitamin A intakes showed that the feeding of the low vitamin E ration did not impair the utilization of carotene and vitamin A.

Some studies on possible procedures for the removal of interference in the chemical assay for tocopherols by the

Emmerie-Engel procedure are reported. These include an alternate procedure for the removal of carotene interference based on the oxidative destruction of the carotene under conditions where α-tocopherol is quantitatively converted to α-tocopherylquinone. The quinone is then converted by reductive cyclization to α-tocopherol and measured as such.

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Dr. J. W. Nelson, Dr. Floyd Olson, Dr. W. W. Kielley and Dr. C. E. Calverley were associated with this project at various times during the period from 1940 to 1946 and are responsible for the miscellaneous blood and tissue and milk composition analyses reported herein, as well as for the control bioassays of the rations used during this period.

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THE MICROBIOLOGICAL ACTIVITY OF VITAMIN B_{12} IN THE URINE OF NORMAL RATS FOLLOWING THE ORAL AND SUBCUTANEOUS ADMINISTRATION OF THIS VITAMIN [1]

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Recently, Chow and others (Chow et al., '50) demonstrated that the oral administration of vitamin B_{12} to normal subjects or to patients suffering from pernicious anemia did not cause the appearance of the microbiological activity of vitamin B_{12} in the urine. On the other hand, after the intramuscular injection of this crystalline vitamin, at least 50% of the original activity of the injected material was found in the urine within 48 hours. These divergent results with respect to the urinary excretion of this vitamin, together with the relatively ineffectual oral therapy of vitamin B_{12} for the treatment of pernicious anemia patients (Berk et al., '48; Hall et al., '49), raise the question of whether the failure of this substance to appear in urine is peculiar only to man, and hence indicates an inability to utilize the vitamin efficiently as an accessory nutritional factor. It is therefore of interest to ascertain whether oral administration of vitamin B_{12} to another species of animal, such as rats, known to be capable of absorbing and utilizing this vitamin (Emerson, '49), will result in its appearance

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[2] The authors are indebted to Mrs. Shanley Davis for her faithful assistance on the microbiological assay of vitamin B_{12}.

in the urine. To this end, we have fed vitamin B_{12} to a series of normal adult rats both orally and parenterally, and followed the appearance of the microbiological activity of this vitamin in their urine. The results are reported in the present communication.

EXPERIMENTAL PROCEDURE AND RESULTS

The effect of route of administration on the urinary excretion of vitamin B_{12}

In the first experiment 6 normal adult rats weighing between 300 and 350 gm were put into individual metabolism cages and offered for three days a soybean diet supplemented with all known vitamins except vitamin B_{12}. Beginning on the 4th day, one 48-hour collection of urine was made. This specimen was taken as a measure of the basal excretion. On the 6th day, one-half of the prepared animals (group A) were given a vitamin B_{12} solution [3] subcutaneously and the remaining half (group B) received it orally. Fifteen milliliters of an isotonic saline solution were also given by injection daily in order to increase the volume of urine excreted and thereby minimize the error due to the volume of urine retained in the bladders of the animals. Collection of urine was continued for 5 additional days.

Five days after the administration of vitamin B_{12} the route of feeding this vitamin was reversed; thus, the group (A) which previously received vitamin B_{12} by the subcutaneous route now received it orally and the group (B) which had received the vitamin orally now received it subcutaneously. Urine was again collected for two additional days. The vitamin B_{12} activity in each specimen of urine was determined with *Lactobacillus leichmannii* 4797 (Skeggs et al., '50).

The results of the first experiment, shown in table 1, demonstrate that the first group (A) of animals excreted approximately three-fourths of the vitamin B_{12} injected parenterally, whereas the vitamin B_{12} content in the urine of the rats in

[3] Squibb Rubramin concentrate.

group B 48 hours after oral administration was not higher than that collected during the basal period for the same time. However, it should be pointed out that the basal excretion for this group of animals was unusually high. When the route of administration was reversed, we again observed that the major portion of the vitamin B_{12} administered subcutaneously appeared in the urine of the rats of the second group (B), and that there was only a small amount of the vitamin in the urine of the rats receiving B_{12} orally. The appearance of some microbiological activity in the urine of these rats may have been due to the contamination of vitamin B_{12} by the stools. Hence, attempts were made to minimize such an error in our second experiment.

This was achieved by replacement of the soybean diet with a 40% sucrose solution and by the removal of fecal matter within one-half hour after defecation during the working day. It was found that with these precautions the variation in the microbiological activity in the urine specimens of the individual rats collected during the two basal periods was much less than that obtained in the first experiment. We believe that this constancy in the basal values can be attributed to the efforts to minimize the contamination of vitamin B_{12} by the stools. The assay results (see table 1) on the specimens of urine obtained after administration of B_{12} demonstrated again that the vitamin B_{12} given by the parenteral route was excreted regardless of the order of administration. On the other hand, following oral administration of as much as 1.0 mg per rat there appeared in the urine a significant quantity of vitamin B_{12} activity, which amounted to 10 to 50 times the basal excretion and to approximately 1% of the administered vitamin. Since there was a very minute amount of fecal matter in the cage, the major portion of the activity in the urine specimens was probably the result of excretion rather than of contamination by stools or of the production of B_{12} by microorganisms present in the urine.

The data further demonstrate that vitamin B_{12} activity continued to be present in the urine as long as 48 to 72 hours after

TABLE 1

The appearance of vitamin B_{12} activity in the urine of normal adult rats after oral or subcutaneous administration

GROUP A				GROUP B			
Route	Hours from administration	Micrograms excreted	Total excretion [1]	Route	Hours from administration	Micrograms excreted	Total excretion [1]
			Experiment 1				
	−48	0.09	0		−48	0.30	0
Subcutaneous (30 μg)	0	...	..	Oral (30 μg)	0	...	..
	7	21.0	70		24	0.28	0
	24	1.6	75		48	0.19	0
	48	0.31	76		120	0.34	0
	120	0.51	78	Subcutaneous (30 μg)	0	0	0
Oral (30 μg)	0	0	0		7	20	67
	24	0.40	1		24	0.25	67
	48	...	..		48	0.18	67
			Experiment 2				
	−48	0.07	..		−48	0.11	..
	−24	0.09	..		−24	0.09	..
Subcutaneous (5 μg)	0	...	..	Oral (1,000 μg)	0	...	..
	8	3.2	64		8	0.60	0.06
	24	0.16	66		24	1.47	0.2
	48	0.11	67		48	5.2	0.7
	72	0.13	68		72	1.0	0.8
	96	0.17	68		96	0.28	0.9
Oral (1,000 μg)	0	...	..	Subcutaneous (5 μg)	0	...	..
	8	0.65	0.06		8	3.6	72
	24	0.63	0.1		24	0.24	76
	48	1.8	0.3		48	0.24	78
	72	0.45	0.35		72	0.20	80
	96	0.18	0.36		96	0.15	80
	120	0.18	0.37				

[1] Expressed as per cent of the total vitamin administered.

oral administration, while most of the injected vitamin B_{12} was excreted in 24 hours or less. However, this difference in the rate of excretion may be partially accounted for by the difference in the dosages administered. The pertinent result of this experiment is that the oral administration of as much as 1,000 μg caused the appearance in the urine of only a very small fraction of the microbiological activity of the vitamin B_{12}. It is therefore of interest to ascertain the level of oral intake which must be given in order to cause the appearance of this activity to a marked degree.

TABLE 2

The appearance of the microbiological activity[1] of vitamin B_{12} in the urine of rats following oral administration at different levels

HOURS FROM ADMINISTRATION	TOTAL DOSAGE ADMINISTERED, IN MICROGRAMS					
	10	30	90	270	1000	10[2]
	Micrograms of vitamin B_{12} activity in urine					
—48	0.09	0.04	0.14	0.13	0.11	0.11
—24	0.05	0.07	0.04	0.04	0.04	0.06
0	...	...	...	...	...	...
8	0.03	0.03	0.06	0.18	3.2–4.3–5.7	8.0
24	0.03	0.04	0.05	0.65	0.4–10.0–0.7	1.4
48	0.08	0.10	0.07	0.50	0.47–10.5–4.5	0.10
72	0.04	0.12	0.07	0.12	0.35–4.43–8.50	0.04
96	0.08	0.08	0.04	0.05	0.10–0.14–0.10	0.05

[1] All activity results shown are expressed in terms of micrograms of crystalline vitamin. B_{12}.

[2] B_{12} was administered subcutaneously.

Level of oral intake of vitamin B_{12} and its urinary excretion

Eighteen normal adult rats weighing 300 to 350 gm were divided into 6 groups of three animals each. The first 5 groups were to receive the following doses of vitamin B_{12} orally: 10 μg, 30 μg, 90 μg, 270 μg, and 1,000 μg per rat; and the 6th group, 10 μg subcutaneously. Like the animals in the second experiment, these rats received a 40% sucrose solution ad libitum. After the collection for two 24-hour periods of the

basal urinary excretion, the specified amounts of the vitamin were given to the respective groups. Collections of urine were made at the following intervals: 8 hours, 24 hours, 48 hours, 72 hours, and 96 hours after administration. The results of the determination of the microbiological activity in the urine are given in table 2.

The data demonstrate that there was no significant increase of the microbiological activity in all the urine specimens from the rats receiving 90 μg or less of B_{12} by the oral route over that in the basal excretion. Hence, it may be concluded that no appreciable amount of the fed material appeared in the urine. On the other hand, when the oral dose was increased to 270 μg, a small amount of vitamin B_{12} was excreted. The excretion of vitamin B_{12} increased as the oral dosage was further increased to 1,000 μg. The results of the microbiological assay of the urine specimens of three individual rats receiving 1.0 mg B_{12} are given because of the large variations among the rats used. In spite of this variation, there is no doubt that this group of animals excreted a considerable amount of vitamin B_{12}. The delayed excretion after the oral administration, which was pointed out in our second experiment, was again noted in this series of rats. As would be expected, the administration of 10 μg of vitamin B_{12} subcutaneously caused the total excretion of the injected material within 24 hours.

Effect of repeated oral administration of vitamin B_{12} on urinary excretion

An experiment was designed to ascertain the effect of repeated oral administration of even higher doses of vitamin B_{12} on the appearance of microbiological activity in the urine. To this end 8 normal rats were offered a soybean diet and then a 40% sucrose solution. The collections made during the first two 24-hour periods were taken as the basal excretion. The animals were then divided into three groups. Group A (4 rats) were given 2 mg of B_{12} orally and another 2 mg 4 hours later; thus the total dosage was 4 mg. Group B (two rats) were

given the identical amount of this vitamin, at the same intervals, by the subcutaneous route. Group C (two rats), which received two injections of a normal saline solution during the same time intervals, served as a control.

The results of microbiological assay (table 3) showed that animals receiving the massive doses of vitamin B_{12} solutions by the subcutaneous route excreted the entire portion of the vitamin in the urine within 24 hours after the first injection, although the microbiological activity continued to appear in the urine, in amounts greatly exceeding the basal value but

TABLE 3

The appearance of vitamin B_{12} activity [1] in the urine of normal adult rats after oral or subcutaneous administration of two massive doses (4.0 mg per rat) at close time intervals

HOURS FROM ADMINISTRATION	GROUP A (Oral route)	GROUP B (Subcutaneous route)	CONTROL GROUP (No B_{12})
	Micrograms of vitamin B_{12} activity in urine		
—48	0.04	0.03	0.07
—24	0.05	0.03	0.04
0	...	...	...
24	65.0	4,050.	0.03
48	71.0	109.0	0.08
120	28.0	72.0	0.38

[1] All activity results shown are expressed in terms of micrograms of crystalline vitamin B_{12}.

equivalent to only a small fraction of the administered dose, even after 120 hours. The animals which received the massive doses orally excreted only a small fraction (about 4%) of the administered vitamin. Assay of the microbiological activity in the urine of rats in the C group demonstrated that the basal excretion of the animals on the sugar diet was essentially constant except for the last period, when it was unusually high. We believe this to have been due to fecal contamination.

These results therefore demonstrate that in spite of the oral administration of two massive doses of vitamin B_{12} at intervals of 4 hours, only a small amount of the microbiologi-

cal activity appeared in the urine; whereas subcutaneous injections of the same amount of this vitamin caused the appearance in the urine of almost the entire amount of the administered vitamin.

DISCUSSION

It is well known that the oral administration of vitamin B_{12} for the treatment of pernicious anemia patients is less effective than the parenteral administration unless the oral administration of this vitamin is accompanied by the gastric juice obtained from normal individuals, or unless the dosage is increased many-fold. These facts may be taken to indicate that only a small amount of the vitamin B_{12} given orally is absorbed. The lack of appearance of vitamin B_{12} activity in the urine of normal subjects or of pernicious anemia patients can also be explained on the basis of poor absorption. It is therefore of interest to ascertain whether similar results can be obtained in another species of animal, such as rats, where it has been demonstrated that the administration of vitamin B_{12} by the oral route is effective for the promotion of growth, reproduction, and other important physiological functions. The results presented in this paper demonstrate that the oral administration of crystalline vitamin B_{12} to normal adult rats, as to man, did not cause the appearance of its microbiological activity in urine if the dosages were no higher than 90 μg per 300 gm of body weight. This dosage is equivalent to about 21 mg for a 70 kg man. The biggest dosage of crystalline vitamin B_{12} which we have so far administered to man is 5.0 mg. At this level, there was no detectable B_{12} activity in the urine.

When the dosage was increased to 270 μg per rat of the same body weight, some microbiological activity appeared in the urine. Further increase of the oral intake by 10- or 15-fold, either as a single dose or as repeated dosages, failed to increase materially the microbiological activity or to prolong greatly the presence of the activity in the urine.

The results presented in this paper therefore demonstrate that the rat, like man, does not excrete appreciable quantities

of vitamin B_{12} in the urine, when it is given orally, unless the dosages are very high. Since feeding vitamin B_{12} to rats, by mouth, in dosages much smaller than those used in our experiment can stimulate growth, reproduction, and other important functions, the absence of the microbiological activity in the urine cannot by itself be taken to indicate an inability to absorb the vitamin.

SUMMARY AND CONCLUSION

Vitamin B_{12} was given to a series of rats both by the oral and subcutaneous routes. Vitamin B_{12} activity was found to be absent in the urine of animals receiving B_{12} orally until a dosage of at least 270 μg/300 gm rat was reached. At this or a higher level, only a small fraction of the administered vitamin appeared in the urine. The administration of crystalline vitamin B_{12} to rats by the subcutaneous route caused the appearance of the microbiological activity of this vitamin in the urine in amounts essentially equal to the amount injected. Since it has been demonstrated that rats can absorb and utilize vitamin B_{12} for normal physiological functions, and since this species of animal, like man, cannot excrete this vitamin following oral administration, the absence of vitamin B_{12} activity in human urine cannot be taken as evidence that the vitamin is not absorbed or utilized by man.

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THE INFLUENCE OF LOW LEVELS OF PROTEIN ON HEAT PRODUCTION [1]

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During recent years a series of experiments have been conducted in this laboratory to study the effects of various levels of protein in equicaloric diets upon the heat production of albino rats (Forbes, Swift, Black and Kahlenberg, '35; Forbes, Voris, Bratzler and Wainio, '38; Forbes, Black, Thacker and Swift, '39, '40; Forbes, Swift, Marcy and Davenport, '44). The most striking observation has been a diminution in the heat production, at a moderate rate, as the protein content of the diet was increased, and in view of the earlier generally accepted theory that the dynamic effect of a diet is determined, in the main, by the protein which it contains, the results presented a new idea in the understanding of dynamic effect.

The series of experiments demonstrating this relationship involved 231 albino rats in 6 individual experiments, each of 70 days' duration. The heat production was measured as a single value for the entire 70-day period by deducting the energy of the excreta and body gain from the gross energy of the food. The nitrogen and energy content of the rat bodies at the start of the experiment were determined by analysis of litter-mate controls sacrificed at the start of the experiment. This method of experimentation offers distinct advantages over many other procedures in that long periods of measurement serve to minimize procedural errors as well

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as to cover a large segment of the normal life cycle of the rat, especially the growth period. An advantage is offered also in that the experimental animals are permitted normal freedom of movement in the metabolism cage.

Further evidence lending support to the general view that combinations of nutrients when mixed in rations do not produce dynamic effects in proportion to the individual nutrients composing the rations has been presented by Forbes and Swift ('44). In that work the dynamic effects of protein (beef muscle), carbohydrate (cerelose) and lard were studied both individually and in various combinations at planes of nutrition above maintenance, the dynamic effect being measured as the difference in heat production on a complete basal diet and on the same diet plus one or more of the supplements. The dynamic effect of the mixed supplements of protein and fat, and of carbohydrate and fat, were even lower than the dynamic effects of the fat. Computed to 1,000 cal. of gross energy of the supplement, the combinations with the highest dynamic effects were protein, carbohydrate and fat; and carbohydrate and protein. In these tests fat was more of a controlling factor in respect to expression of dynamic effect than either protein or carbohydrate. The shortcomings involved in attempting to estimate the dynamic effect of mixed diets from individual data become increasingly obvious after consideration of such results.

The purpose of the present experiment was to extend the study of the influence of various levels of protein on the heat production of albino rats to include levels of protein lower than had been fed at any previous time in this series of investigations. When the original work was planned it was anticipated that the curve of heat production would vary more or less directly with the protein intake, but when it was found that the highest level of heat production was obtained with the lowest protein intake, which was 10% of the diet, the need to study levels of protein lower than this became evident.

EXPERIMENTAL

In the present experiment three groups of male albino rats were used, each group being composed of 12 animals of approximately the same weight selected as litter-mate triplets, one animal from each set of triplets being placed in one of the three main groups. Each of the groups was given one of the experimental rations, these being compounded to contain approximately 6, 8 or 10% protein and made equicaloric by

TABLE 1

Composition of diets[1]

CONSTITUENTS	DIET		
	1	2	3
	%	%	%
Cellu flour	4.00	4.00	4.00
Salts IV[2]	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
Butterfat	1.00	1.00	1.00
Yeast[3]	6.00	6.00	6.00
Dextrin	63.11	61.18	59.26
Dried whole egg	7.79	12.24	16.69
Crisco	13.10	10.58	8.05
Total	100.00	100.00	100.00

[1] Crystalline carotene was added to each diet to provide the equivalent of 1.4 I.U. of vitamin A per gram.

[2] Lichstein et al. ('46).

[3] Yeast was a mixture of 5 parts brewers' yeast and one part irradiated yeast.

variation in the amounts of carbohydrate and fat. The vitamin and mineral constituents were constant in all three rations and for comparative purposes were kept at the same levels as in former experiments of this series. The exact composition of the diets may be found in table 1.

The method of feeding was by the paired technique with modification to include three animals in a group instead of two, the animal consuming the least food in any triplicate determining the food consumption for the triplicate.

Details of the conduct of this type of investigation may be found in the report of the experiment where it was used first, by Swift, Kahlenberg, Voris and Forbes ('34).

In the previous work of this series the source of protein was either casein or dried beef muscle, but since the plan in the present experiment was to feed such a low level of protein, it was decided to use one of very high biological value, and for this reason dried whole egg was selected.

The lowest level of protein to be fed, 6%, was decided in a more or less arbitrary manner. As some growth was considered desirable, an attempt was made to select a diet where

TABLE 2

Average amounts of food eaten and gains in live weight, nitrogen and fat per rat during 70 days

PROTEIN IN DIET	FOOD EATEN, DRY MATTER	LIVE WEIGHT		GAIN IN EMPTY BODY WEIGHT [2]	NITROGEN OF BODY GAIN	FAT GAINED
		Initial	Final			
%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
6.19	424	48.2 ± 0.3 [1]	121.9 ± 1.9	76.0 ± 1.9	2.26 ± 0.1	14.43 ± 0.6
7.94	425	48.2 ± 0.4	150.0 ± 3.0	104.0 ± 3.0	3.29 ± 0.1	17.85 ± 0.8
10.00	426	48.1 ± 0.3	167.8 ± 3.6	121.6 ± 3.6	4.09 ± 0.1	18.25 ± 1.1

[1] Probable error of the mean.

[2] Contents of alimentary tract removed.

this would still be possible but at the same time to feed a level of protein as low as would be of practical importance.

Reasonable growth was obtained on the 6% protein intake, being slightly more than 1 gm per day on the average. Growth on the 8 and 10% protein levels was determined largely by the limitation of feed imposed on these rats by the animals on the 6% protein intake, as it was this latter group that determined food consumption. The group receiving the 6% protein ration had 437 refusals of feed, while the 8% protein rats had 43 refusals and the 10% protein group 21 refusals. Twenty-one of the refusals occurred in the 8 and 10% protein

rats on the first day of the experiment, which was due largely to the abrupt change in diet.

In table 2 are given the average amounts of food eaten, gains in live weight and gains in nitrogen and fat per rat for the 70-day experimental period. All these values increased with the increase in the protein content of the diet. From a statistical standpoint the odds, computed by Student's method, are well over 10,000 to 1 that the gains in nitrogen are significant with each increase in nitrogen intake. While the gains in fat show an average increase of reasonable magnitude, there was considerable variation among individual animals,

TABLE 3

Distribution of food nitrogen as affected by the plane of protein intake

PROTEIN IN DIET (%)	6.19	7.94	10.00
Nitrogen:			
Food (gm)	4.52	5.80	7.31
Feces[1] (gm)	1.00 ± 0.0[2]	1.08 ± 0.0	1.19 ± 0.0
Digested (gm)	3.52	4.72	6.12
(Apparent)			
% of food N	77.9 ± 0.2	81.4 ± 0.2	83.7 ± 0.1
Urine (gm)	1.22	1.53	2.01
% of food N	27.0 ± 0.4	26.4 ± 0.6	27.5 ± 0.6
Gain of N (gm)	2.26 ± 0.1	3.29 ± 0.1	4.09 ± 0.1

[1] Includes endogenous nitrogen.

[2] Probable error of the mean.

with the result that the odds are much less (276 to 1) that the 8% protein rats gained more fat than the 6% protein rats, and 224 to 1 that the 10% rats gained more than the 6% rats.

Data on nitrogen utilization are shown in table 3. Both the fecal and urinary nitrogen increased as the protein increased, which is in accord with general understanding on this point when rations containing these quantities of egg proteins are fed. One matter of importance, although it is not shown in the table, is the recovery of the nitrogen of the food in the feces and urine and the body gain. This average value for the 70-day experimental period was 100.3%, the lowest re-

covery being 96.8% and the highest 103.8%. This is a highly satisfactory result when the limits of experimentation are considered.

The decreased digestibility of nitrogen at the lower levels of protein intake apparently reflects, to a large degree, the fact that metabolic nitrogen accounts for a relatively high percentage of the total nitrogen of the feces.

Distribution of the total food energy is given in table 4. The excretion of energy in the feces and urine is essentially the same on all three diets, resulting in similar amounts of

TABLE 4

Distribution of total food energy as affected by the plane of protein intake

PROTEIN IN DIET (%)	6.19	7.94	10.00
Energy value in Calories			
Food	2128	2128	2128
Feces	204 ± 8 [1]	199 ± 5	190 ± 5
Digested	1924 ± 44	1929 ± 47	1938 ± 46
Urine	16 ± 1	18 ± 1	20 ± 1
Metabolized	1908 ± 44	1911 ± 46	1918 ± 46
Body gain			
total	216 ± 7	287 ± 11	319 ± 13
as protein	82 ± 2	121 ± 4	149 ± 4
as fat	134 ± 5	166 ± 8	170 ± 10
Heat production	1692 ± 38	1624 ± 38	1599 ± 36

[1] Probable error of the mean.

metabolizable energy. The total gains of energy and of protein as body tissue show a very significant difference in direct relation to the protein intake. Due to greater individual variation, the significance is greatly reduced in the comparisons of energy gained as fat, but the odds are still relatively high (250 and 290 to 1) that the 8 and 10% protein rats, respectively, gained more energy in this manner than the 6% protein rats.

The highest heat production was found in this experiment, as in former studies, in the rats receiving the lowest level of protein. From a statistical standpoint, the odds that the 6%

protein rats produced more heat than the 8% protein rats are well over 10,000 to 1, are even higher for the comparison of the 6% and the 10% protein rats, but are little more than even that the 10% protein rats produced more heat than the 8% protein rats. With such small differences in protein content among the rations (2%), it is not surprising that a high degree of significance in heat production cannot in all cases be demonstrated between successive levels of protein intake.

In all comparisons in this series of experiments using equicaloric diets varying in protein content from 6 to 45%, the heat production has decreased in each comparison as the protein has been increased. The sources of protein used have been without apparent influence on the results obtained.

SUMMARY

Study of the influence of various levels of protein in equicaloric diets on protein and energy metabolism has been extended in the present experiment to include very low levels of protein, 6 previous studies having covered the range of protein intake from 10 to 45%.

Using a 70-day metabolism and body analysis technique with three groups of 12 litter-mate, growing albino rats, a comparison was made of three diets containing 6, 8 and 10% protein, respectively, these diets being so compounded and fed as to supply to each rat of a set of triplets the same gross energy, vitamins and minerals. The heat production was measured as a single value for 70 days by subtracting the energy of the excreta and body gain from the gross energy of the food.

Gains in live weight, retention of nitrogen and fat in body tissue, increased digestibility of nitrogen, and decrease in heat production were in the order of increasing protein content of the diet.

The principal demonstration of this entire series of studies is a decrease in heat production at a moderate rate as the protein in equicaloric rations increases from 6 to 45%.

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METHIONINE DEFICIENCY UNDER AD LIBITUM AND FORCE-FEEDING CONDITIONS[1]

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ONE FIGURE

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The effects of incomplete amino acid mixtures on nitrogen retention by animals have been studied by many workers employing the classical nitrogen balance technique of Mitchell described in 1924 (Maksimova, '36; Wolf and Corley, '39; Burroughs et al., '40; Allison et al., '47; Brush et al., '47; Cox et al., '47; Wissler et al., '48; Rose et al., '50; Wykes et al., '50).

Maksimova ('36) observed that when tyrosine, cystine and tryptophan were added to a protein-free ration fed to dogs, an increase in nitrogen retention by the animals ensued. Wolf and Corley ('39), studying the effects of incomplete amino acid mixtures on nitrogen balance in adult female rats fed diets low in nitrogen, observed that the omission of any one of the 9 essential amino acids resulted in a negative nitrogen balance. Wissler et al. ('48) observed the same effect when a ration containing a higher level of nitrogen was fed to adult, male rats.

The differing results which have appeared in the literature concerning nitrogen balance in rats fed diets deficient in cer-

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tain amino acids may possibly be explained by differences in the level of nitrogen intake as well as the amino acid composition of the ration. The nitrogen intake may be reduced to a level at which small decreases will cause animals to go into a negative nitrogen balance whether the nitrogen output changes or not. On the other hand, it is conceivable that the nitrogen intake could be raised to a level at which it would be exceedingly difficult for an animal to excrete more nitrogen than is consumed.

A survey of the literature reveals that the food intake of animals receiving an amino acid-deficient ration ad libitum may be highly variable, and usually decreases with the length of time on the deficient ration. Since both variability in nitrogen intake and inanition may interfere with the specific effects of an amino acid deficiency, a force-feeding procedure may be undertaken in order to control the food intake rigidly. Under such conditions the effects due to an amino acid deficiency would be obtained without interference from variation in food intake.

When conducting experiments to observe the effects of a methionine deficiency upon amino acid metabolism in the growing rat, it was noted that the animals force-fed a methionine-deficient ration remained in positive nitrogen balance throughout the two-week feeding period. Since it has been shown that low levels of nitrogen intake by rats cause a decrease in the xanthine oxidase activity in the liver (Miller, '48; Williams and Elvehjem, '49), it was desirable to feed relatively high levels of nitrogen to keep the control animals healthy. These unexpected results then led us to study the effects of ad libitum food intake. These findings were compared with those obtained from the force-fed rats, where the nitrogen level was kept normal throughout the feeding experiment.

EXPERIMENTAL AND RESULTS

Male, albino rats of the Holtzman strain with initial weights of approximately 60 gm were used as experimental animals.

Two methods of feeding were employed throughout the two-week feeding period. The animals in group I, consisting of 12 rats, were given the rations by a force-feeding procedure, while those in group II, consisting of 8 rats, were fed ad libitum, employing the method of Wykes et al. ('50). A methionine-deficient ration was fed to 6 of the animals in group I and to 4 of the animals in group II. The remaining rats in each group were given the complete ration.

The animals used for the ad libitum feeding experiments were placed in metal metabolism cages with wire tunnels attached to openings in the sides of the cages; this allowed passageway to the ration, which was placed in containers constructed with wire screening (no. 4 mesh) covering the ration. The ration was accessible only through a circular hole just large enough for the rat's head and this arrangement allowed the animals to reach the ration only by pushing their tongues between the spaces in the screen (tongue-feeding). With this procedure, contamination of the urine and feces due to the dropping of particles of the ration from fur and paws was kept at a minimum. The animals which were used in the force-feeding experiment (group I) were placed in the same type of cages with the tunnels blocked off. They were force-fed a complete, purified ration for three days prior to the feeding experiments, which allowed the rats to become accustomed to the force-feeding procedure; by gradually increasing the amount force-fed to the desired level, diarrhea and bloating could be prevented. Since these difficulties were not encountered in the group fed ad libitum (group II), an acclimatization period did not appear to be necessary for these animals

The complete ration used in this experiment was the one found best by Ramasarma, Henderson and Elvehjem ('49) and had the following percentage composition: non-essential amino acids 7.9, essential amino acids 8.7 [2], L-arginine monohydrochloride 0.5, glutamic acid 2, corn oil 5, salts IV (Phillips and Hart, '35) 4, monobasic sodium phosphate monohydrate

[2] DL-leucine was substituted at twice the level for L-leucine.

0.63, dry vitamin mix [3] 2, and enough sucrose to make 100. The methionine-deficient ration had the same composition as the one described previously except that sucrose was substituted for methionine. Water was given ad libitum to all animals throughout the feeding period.

Force-feeding was accomplished by homogenizing the ration in water to make a slurry and administering it by stomach tube. Since it had previously been found that the average food consumption of 60-gm rats receiving the complete, purified ration ad libitum was approximately 6 gm per day, this amount was force-fed daily in three feedings at 5-hour intervals. Food consumption data for the animals fed ad libitum were recorded at the termination of each three-day period.

All urines were collected under toluene acidified with hydrochloric acid. The samples were collected every three days and in some cases the urines from two animals were pooled. The urine samples were then diluted to a definite volume for total nitrogen determinations.

Fecal collections were made at the same time as the urine collections. The feces were covered with alcohol acidified with two drops of concentrated hydrochloric acid, and then dried. After drying and weighing, the samples were ground in a mortar and portions were taken for nitrogen determinations.

The nitrogen determinations were made on the aliquots of urine and fecal samples, using a modified, semi-micro Kjeldahl method. When an animal died of methionine deficiency, urine and fecal nitrogen determinations were made to observe any changes which might have occurred during the last day on experiment.

In order to facilitate correlation of the weight changes, nitrogen intakes, and nitrogen balances, the results of both

[3] Each 2-gm portion of the vitamin mixture in sucrose base contained 0.2 mg thiamine hydrochloride, 0.3 mg riboflavin, 0.25 mg pyridoxine hydrochloride, 2 mg calcium d-pantothenate, 1.5 mg niacin, 100 mg choline chloride, 0.01 mg biotin, 0.02 mg pteroylglutamic acid and 10 mg inositol. Two drops of fortified halibut liver oil were administered each week by dropper.

the force-feeding and ad libitum feeding experiments are presented in graph form in figure 1 (I–VI).

The weight curve, figure 1 (I), of the animals force-fed the complete ration indicates that there was an immediate gain in weight by these animals after the three-day acclimatization

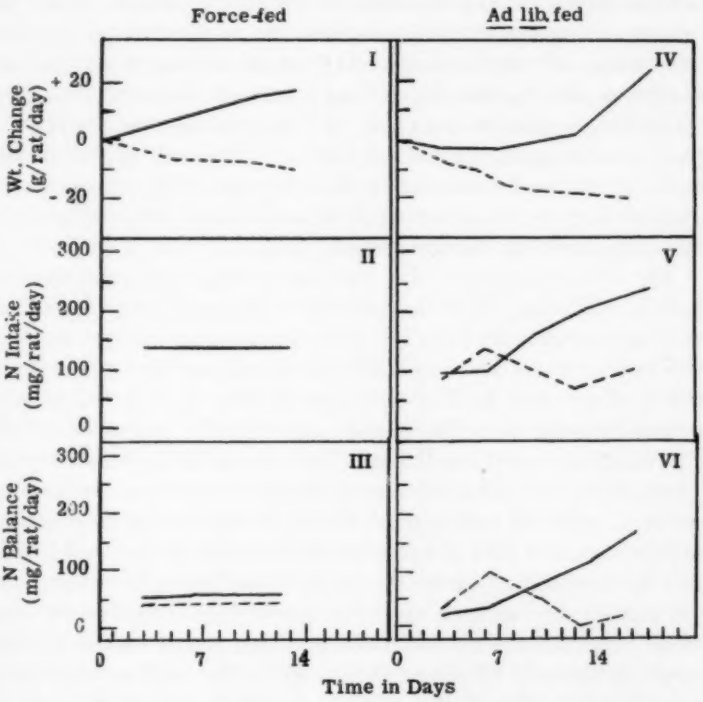

Fig. 1 Weight change, nitrogen intake and nitrogen balance curves of animals receiving a complete or methionine-deficient ration ad libitum or by force-feeding. - - - - - = methionine-deficient animals. ——— = control animals.

period. On the other hand, the weight curve, figure 1 (IV), of the animals receiving the complete ration ad libitum does not indicate a weight increase until about 7 days after the start of the feeding experiment. A similar lag in growth was observed by Ramasarma et al. ('49) when feeding the same purified

amino acid mixture. By comparing the nitrogen intake curve in figure 1 (V) with the weight gain curve in figure 1 (IV) of the animals fed the complete ration ad libitum, it can be seen that the poor growth exhibited the first few days was probably due to the low amount of ration consumed. This low food intake occurred during the time in which the animals were becoming accustomed to the ration and to the tongue-feeding procedure. A comparison of the weight curves of the groups receiving the methionine-deficient ration indicates that the force-fed groups of rats did not lose weight as rapidly as those receiving the ration ad libitum. This was probably due to the lower food intake by the latter group. The weight losses exhibited by the methionine-deficient animals were similar to those observed by Womack et al. ('37).

The nitrogen intakes for the two groups are presented in figure 1 (II, V). Since the animals in group I were force-fed, the food intakes for both the methionine-deficient and the control group were the same and remained constant throughout the feeding period. The nitrogen intakes by the animals in group II were, as can be seen in figure 1 (V), highly variable. The animals receiving the complete ration had a tendency to consume more food after becoming accustomed to the feeding regimen. On the other hand, those receiving the methionine-deficient ration had a much more variable daily food intake but it remained at about the same level throughout the feeding period. It has been observed previously that the omission of an essential amino acid from a ration results in an impairment of appetite (Frazier et al., '47). The degree of impairment may be associated with a "protein depletion" period (Wissler et al., '48). Our animals did not exhibit a marked decrease in appetite, although the food consumption of the animals in group II receiving the methionine-deficient ration was much lower than that of the controls in the same group.

The nitrogen balance curves plotted in figure 1 (III, VI) exhibit a striking similarity to the nitrogen intake curves in the same figure (II, V). The nitrogen balance curves of the animals in group I, and also the nitrogen intake curves, re-

mained constant throughout the feeding period. The slight difference betwen the two curves from the force-fed groups cannot be considered significant, since this difference prevailed at the start of the feeding period (not shown in the curves). The nitrogen balance curves of the animals fed ad libitum tended to be variable and to follow the same trends as the nitrogen intake curves. Since the amount of nitrogen excreted daily by each animal remained fairly constant throughout the feeding period, fluctuations in the nitrogen balance curves were probably due primarily to the varying nitrogen intakes. For example, the nitrogen intake of the animals in group II receiving the methionine-deficient ration increased during the second three days of the experiment and, as can be seen in figure 1 (V), the nitrogen balance curves of these rats increased during the same period.

The prolonged positive nitrogen balance period exhibited by the animals in both groups receiving the methionine-deficient ration may appear contradictory to the reports of previous workers (Wolf and Corley, '39; Nielsen and Corley, '39; Burroughs et al., '40; Wissler et al., '48) who have observed the effects of methionine on nitrogen retention by adult rats; however, the animals fed the methionine-deficient ration ad libitum had a tendency to go into a negative nitrogen balance during the latter part of the experimental period. This prolonged period of positive nitrogen balance exhibited by the methionine-deficient animals in both groups may possibly have been due to the fact that these animals were not placed on a nitrogen-low ration prior to the feeding studies. More probably, however, it was due to the fact that the daily intake of nitrogen remained high throughout the feeding period. Although Rose et al. ('50) have shown that the removal of methionine from the human diet results in an abrupt negative nitrogen balance, the work of Allison et al. ('47) with dogs and that of Burroughs et al. ('40) with rats indicates that there is approximately a 4-day time interval before the effects of the removal of methionine can be detected by nitrogen excretion studies.

Brush et al. ('47) have suggested that methionine may serve mainly in the body in some capacity other than that of building body proteins. If the experimental animals are subjected to a nitrogen-low feeding period prior to feeding a ration deficient in methionine, then, as Brush et al. observed, methionine, because of its suggested role in the diet, would exhibit a marked nitrogen-sparing action. On the other hand, if the animals are not subjected to a "depletion" period, they may be able to obtain enough methionine from the breakdown of body protein to fulfill partially its specific function and thereby allow the animal to retain part of the exogenous nitrogen.

A methionine-deficient condition was manifested in the animals by an emaciated appearance, bloody mouth and paws and poor growth. Approximately half of the animals in both groups receiving the methionine-deficient ration died within the two-week feeding period. A severe weight loss was encountered by the methionine-deficient animals in both groups; however, that exhibited by the group fed ad libitum was greater than that of the force-fed group. The animals in the force-fed group died without excreting a higher amount of nitrogen than those that remained alive; consequently, they did not go into negative nitrogen balance. It appears, then, that under the conditions of this experiment the ability of growing animals force-fed a methionine-deficient ration to retain nitrogen is apparently not affected by such feeding. Although the animals receiving the methionine-deficient ration ad libitum showed a tendency to go into a ngative nitrogen balance during the latter part of the feeding period, this may possibly have been due to the continued low intake of food.

It has been shown that under the conditions of these experiments both the nitrogen intake and nitrogen balance curves of rats receiving a ration containing 19.1% amino acids follow the same trends. Therefore, in considering the effects of an amino acid deficiency upon nitrogen retention by growing animals, it appears imperative that one consider the level of nitrogen in the ration as well as the amount of ration consumed.

SUMMARY

The effects of methionine deficiency on weight, nitrogen intake, and nitrogen balance have been observed employing both ad libitum and force-feeding procedures.

Under the conditions of this experiment there was a tendency for the weights of the animals receiving the ration ad libitum to differ from those of the group receiving the ration by a force-feeding procedure. This can probably be attributed to differences in the amounts of ration consumed.

Since the nitrogen balance curves and the nitrogen intake curves followed almost exactly the same trends, it appears that fluctuations in the nitrogen balance curves were due to the amount of ration consumed. This is substantiated by the fact that a fairly constant daily excretion of nitrogen was observed; therefore, variations in nitrogen intake will cause fluctuations in the nitrogen balance curves.

Comparing the results of our studies with those of other workers, it appears that the level of nitrogen in the ration, as well as the actual amount of food consumed, must be considered when observing the effects of an amino acid deficiency on nitrogen retention.

ACKNOWLEDGMENT

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AN UNIDENTIFIED FACTOR PRESENT IN HOG INTESTINAL MUCOSA REQUIRED BY THE MINK [1]

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TWO FIGURES

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It has been demonstrated that mink given a purified ration require two unidentified liver factors for survival (Schaefer, Tove, Whitehair and Elvehjem, '48). One of these factors was found to be soluble in 60% methanol (methanol extract factor) and the other was insoluble in this solvent (residue factor). In an effort to determine whether the nutritional needs of this animal could be met by liver supplementation, mink were maintained on this regimen for a longer time than in the experiments previously reported. The data presented here show that the mink requires an additional factor present in hog intestinal mucosa.

EXPERIMENTAL AND RESULTS

Thirty-three adult male mink were used in these studies. They were housed and handled in the same manner as in earlier experiments. The basal ration consisted of sucrose 66 gm,

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were supported by project 614 of the Wisconsin Agricultural Experiment Station, and by grants from the Borden Company, New York, New York, and Fur Farm Food and Supplies, St. Paul, Minnesota. The work was reported briefly in Fed. Proc., *9*, 373, 1950.

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casein [3] 19 gm, cottonseed oil 11 gm, and salts IV (Phillips and Hart, '35) 4 gm. Each 100 gm of ration were supplemented with 0.2 mg thiamine HCl, 0.2 mg pyridoxine HCl, 0.4 mg riboflavin, 1.5 mg Ca pantothenate, 4.0 mg nicotinic acid, 100 mg choline, 25 mg *i*-inositol, 50 mg *p*-aminobenzoic acid, 0.5 mg 2 methyl 1-4 naphthoquinone, 0.1 mg pteroylglutamic acid and 0.025 mg biotin. Haliver oil fortified with α-tocopherol acetate and vitamin D_3 was added so that 100 gm of ration con-

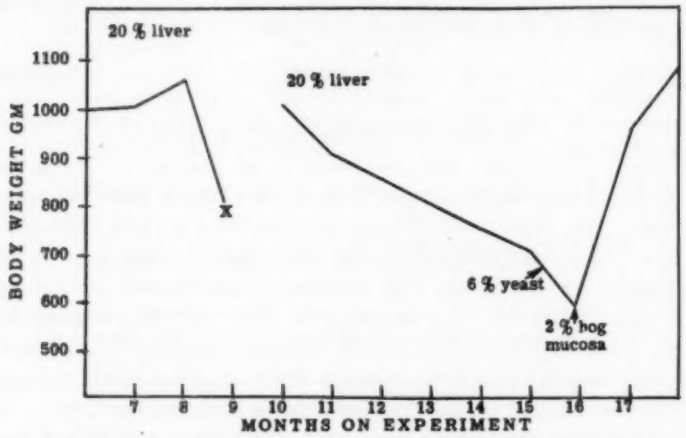

Fig. 1 Body weight curves of mink 96 and 77. Mink 96 received 20% liver from the start of the experiment and died. Mink 77 received 20% liver; 6% yeast was added when indicated. Two weeks later the yeast was replaced by the hog intestinal mucosa.

tained 1,200 I.U. vitamin A, 120 I.U. vitamin D and 4 mg α-tocopherol acetate.

Even though the mink are given the basal ration supplemented with 20% fresh liver, loss of weight, anorexia, matted fur and death will occur after about 10 months on experiment. The body weight curve of mink 96, representative of an animal which died on such a regimen, is given in figure 1. The body weight curve of mink 77 is also presented in figure 1. The ad-

[3] Smaco.

dition of 6% dried brewers' yeast to the liver-supplemented ration of this animal failed to reverse the decline in weight.

When, however, 2% desiccated hog intestinal mucosa was added, a growth response of 525 gm in 12 weeks was obtained. In addition to the results obtained with this animal, results with mink 95, 118 and 181 show that a rapid increase in body weight occurs when 2% desiccated hog intestinal mucosa is added to the basal ration supplemented with liver (table 1).

The effect of the various liver fractions and hog intestinal mucosa on the average survival time of mink receiving the basal ration is given in table 2. It will be noted that the addition of either of the two previously reported liver factors increased the average survival time about one and one-half months. If both fractions of liver are fed, the longevity is greater than when only one is given, but premature death still occurs. When, however, liver and hog intestinal mucosa are given, the mink will live for more than two years. After two years on experiment some of these animals were sacrificed and autopsied.

Mink which died while receiving either fresh liver or the combined methanol extract and residue portions of liver were autopsied, and extreme fatty degeneration of the liver, kidneys and myocardium was noted. Furthermore, enlargement of the right side of the heart and atrophy of the adrenal cortex were often observed. In contrast, the heart and adrenals of animals fed liver and hog mucosa were normal, but a slight fatty degeneration of the liver was still apparent. The kidneys of these animals contained small pin-point spots of scar tissue, indicating that a healing process had occurred.

When mink 181 (table 1), receiving the basal ration plus the methanol soluble and insoluble portions of liver (equivalent to 10% liver) and 2% hog intestinal mucosa, began to lose weight, the supplements were replaced by 10% fresh liver to insure that the methanol extract and residue factors had not been lost. Body weight continued to decline, and after three weeks 2% desiccated hog intestinal mucosa was added to the ration. An immediate response occurred, and the an-

TABLE 1

The effect of supplements to the basal ration on the body weight of mink

MINK NO.	BEFORE SUPPLEMENT			AFTER SUPPLEMENT		
	Ration	Total weeks on expt.	Body weight	Supplement	Total weeks on expt.	Body weight
			gm			gm
77	Basal + 20% liver	67	660	6% Yeast	69	585
		69	585	2% Hog intestinal mucosa	81	1,105
95	Basal + 8% res. + 2% MeOH ext. + 6% yeast	52	850	20% Liver + 6% yeast	59	675
		59	675	2% Hog mucosa + 20% liver + 6% yeast	66	995
118	Basal + 6% yeast + 10% liver	42	925	2% Hog intestinal mucosa	56	1,400
181	Basal + 1% MeOH ext. + 4% res.	21	1,050	2% Hog intestinal mucosa	30	980
	Basal	30	980	10% Liver	33	710
		33	710	10% Liver + 2% hog mucosa	43	1,250
178	Basal + 1% MeOH ext. + 4% res.	90	915	2% Hog intestinal mucosa	91	775 [1]
210	Basal + 1% MeOH ext. + 4% res.	40	775	2% Hog intestinal mucosa	42	640
216	Basal + 1% MeOH ext. + 4% res.	35	1,205	2% Hog intestinal mucosa	41	1,025
143	Basal	31	800	10% Spleen	32	630 [2]
		32	630	10% Spleen + 2% hog mucosa	37	1,005
116	Basal + 6% yeast + 1% MeOH ext.	47	860	10% Spleen + 2% hog mucosa	55	1,425
		55	1,425	10% Spleen + 2% hog mucosa	58	1,100
		58	1,100	10% Spleen + 2% hog mucosa + 4% res.	70	1,540 [2]
138	Basal	40	650	10% Spleen + 2% hog mucosa	41	510
		41	510	1% MeOH ext.	44	875
187	Basal	20	710	10% Spleen + 2% hog mucosa	22	695
		22	695	1% MeOH ext.	25	860
124	Basal + 6% yeast + 1% MeOH ext. + 4% res.	41	900	Changed to 10% liver	44	850
	Basal	44	850	10% Liver + 2% hog mucosa (autoclaved)	49	800 [1]
133	Basal + 10% liver + res.	31	1,040	2% Hog mucosa (autoclaved)	46	760 [1]

[1] Died.
[2] Had previously responded to methanol extract factor.
[3] Vomit exhibited from eating entire contents.

imal gained 510 gm in 10 weeks. As in the case of mink 181, the addition of hog intestinal mucosa to the basal ration supplemented with the methanol soluble and insoluble portions of liver failed to produce a response in mink 178, 210 and 216 (table 1).

Since the hog intestinal mucosa was apparently ineffective unless given with fresh liver, some other products was sought which would be active with hog mucosa but deficient in the methanol extract and residue factors. Results with mink 143 and 116 (table 1) show that a combination of 10% fresh pork spleen and 2% desiccated hog intestinal mucosa produces a

TABLE 2

The effects of liver fractions and hog intestinal mucosa on the survival time of mink

SUPPLEMENT	NUMBER OF MINK	SURVIVAL TIME
		months
None	26	5.5
MeOH extract	15	7.1
Residue	8	6.9
MeOH extract + residue	10	10.8
Liver	10	9.7
Liver + hog intestinal mucosa	4	> 24

body weight response similar to that obtained with liver and hog mucosa. The inability of fresh pork spleen alone to produce a weight response is illustrated by mink 143.

The increase in body weight obtained in mink 116 with spleen and hog mucosa was not sustained, and a second decline in body weight followed. The body weight response obtained upon the addition of the liver residue indicates that the combination of spleen and hog mucosa does not contain the residue factor. The results with mink 138 and 187 (table 1) indicate that the spleen and hog mucosa combination is also deficient in the methanol extract factor. In both of these animals, which were receiving the unsupplemented basal ration,

the administration of 10% pork spleen and 2% hog intestinal mucosa failed to reverse the rapid decline in weight observed. When, however, the methanol extract was given a prompt response occurred.

The inability of the hog intestinal mucosa factor to withstand autoclaving for 15 minutes at 15 lb. pressure is shown by the data obtained with mink 124 and 133 (table 1). The diets of these mink had been supplemented with liver and auto-

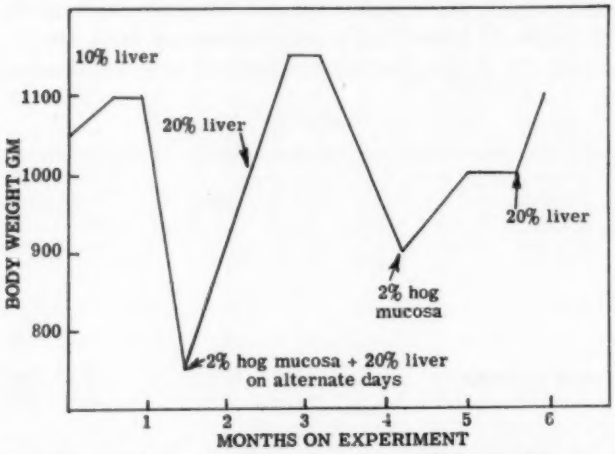

Fig. 2 Body weight curve of mink 209. This animal received 10% liver from the start of the experiment. At the point indicated this supplement was replaced by 20% liver every other day and 2% hog intestinal mucosa on the alternate days. The hog intestinal mucosa was later removed, but the liver supplement continued. After the second decline in weight the liver was replaced by the hog mucosa and later the hog mucosa was replaced by liver.

claved hog intestinal mucosa for at least a month before sudden death occurred, and when these animals were autopsied the typical deficiency syndrome was noted.

The body weight curve of mink 209 is shown in figure 2. The animal was receiving the basal ration supplemented with 10% liver, and after an unusually short time on experiment suffered a marked loss of body weight. When the weight declined to 755 gm, the liver feeding was changed from 10% every day

to 20% every other day and 2% desiccated hog intestinal mucosa was added to the basal ration on the alternate days. The two supplements were given in this manner to insure that the liver and hog mucosa would not come into contact with each other in the gastrointestinal tract. A prompt response in body weight occurred, and after three weeks on this regimen the hog mucosa was withdrawn but the liver continued in the same manner. A second decline in weight followed; when a loss of 200 gm had occurred, the liver was withdrawn, and after an interval of one day the hog mucosa was given. Body weight increased, but the response was not sustained. The hog mucosa was replaced by 20% liver every other day and, as before, the unsupplemented basal ration was given for the intervening day to insure that the two test supplements did not come into contact. This change resulted in an 85-gm increase in body weight in one week.

DISCUSSION

It is evident from the data presented that mink will not survive when given a sucrose-casein purified ration, even though it is supplemented with 20% fresh liver. Autopsy of the animals which die on this regimen reveals an extreme fatty degeneration of the liver, kidneys and heart. It is also apparent that 2% desiccated hog intestinal mucosa supplies a factor, or factors, which cures this syndrome and greatly increases the longevity of the animals on experiment. Since this factor is not found in liver, yeast and spleen, it is unique, in that it is a factor of somewhat restricted source. It is also interesting to note that the hog intestinal mucosa does not produce a response unless given with fresh liver or fresh spleen.

There are two possible means by which the liver-hog mucosa combination exerts its effect: (1) The liver or spleen contains a factor (not present in either the methanol extract or the residue liver fractions) and the hog intestinal mucosa contains an additional factor; (2) the liver or spleen is potentiated by the hog mucosa in a manner similar to that of the intrinsic and extrinsic factors of pernicious anemia. The rather

restricted source of the hog mucosa factor, its lability to heat and its inability to produce a response unless given with fresh liver or fresh spleen, would tend to suggest that it is of an enzymatic nature and hence support the second hypothesis.

The results with mink 209, however, tend to disprove this postulate, for a response was obtained when the supplements did not come in contact with each other. The existence of two factors is shown by the fact that the response obtained with either liver or hog intestinal mucosa alone is not sustained. Since this critical experiment has been performed on only one animal, the results must be considered preliminary.

Recent investigations (Hall et al., '50) have established that hog intestinal mucosa obtained from the duodenal area contains Castle's intrinsic factor. The inactivity of the hog intestinal mucosa for the mink when given with the methanol soluble and insoluble portions of liver, and the effectiveness of feeding liver and hog mucosa on alternate days, suggest that the two hog intestinal mucosa factors are not identical.

In view of the fact that spleen and hog intestinal mucosa do not replace either the methanol extract or residue, it is evident that at least three unidentified factors are required by the mink. Preliminary evidence reveals that a 4th factor is also required. The factors may then be listed as follows: (1) methanol extract factor; (2) residue factor; (3) hog intestinal mucosa factor; (4) spleen or liver factor (not present in the methanol extract or residue fractions of liver).

SUMMARY

1. Loss of weight, anorexia, matted fur and death will occur in mink receiving a purified ration supplemented with liver. Autopsy of these animals reveals fatty degeneration of the liver, kidneys and myocardium. Occasionally enlargement of the right side of the heart and atrophy of the adrenal cortex are also observed.

2. A heat labile factor present in desiccated hog intestinal mucosa cures the syndrome of this deficiency and increases the survival time of the mink on experiment.

3. Preliminary evidence indicates that another factor present in fresh spleen and fresh liver is also required.

4. Neither of these factors is identical with the previously reported methanol extract and residue factors.

ACKNOWLEDGMENTS

We wish to acknowledge our indebtedness to Merck and Company, Rahway, New Jersey, for the crystalline vitamins; to Lederle Laboratories, Inc., Pearl River, New York, for the pteroylglutamic acid; and to the Wilson Laboratories, Chicago, Illinois, for the hog intestinal mucosa.

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NITROGEN, METHIONINE AND CYSTINE CONTENT OF HEN'S EGGS. THEIR DISTRIBUTION IN THE EGG WHITE AND YOLK [1]

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INTRODUCTION

The possibility of changing the amino acid content of a hen's egg by changing the hen's feed has been shown (Csonka et al., '47a). In those experiments we chose casein and soybean proteins to convert our basal low-protein diet to a high-protein casein and high-protein soybean diet. Both of these high-protein diets resulted in eggs with higher cystine and methionine content than eggs of the same hens fed a basal low protein feed in which corn meal furnished the main protein. In the present paper the influences of dietary protein, and also the effect produced by the aging of the pullet, on the nitrogen, cystine and methionine content of the hen egg are discussed. To ascertain wherein the chief increase of cystine and methionine occurred, the egg white and the yolk were analyzed separately.

We have shown earlier that feeding DL-methionine as a dietary supplement does not change the cystine or methionine content of the egg (Csonka et al., '47a). As an explanation for this finding we suggested the difference that exists in rate of

[1] This is the second article in a series dealing with the influence of dietary protein. A preliminary report was presented by the author before the Federation of American Societies for Experimental Biology, 1950 annual meeting, held at Atlantic City, New Jersey. Fed. Proc., Part 1, *9:* 164 (1950).

absorption between the free and the bound amino acids. Experiments are presented here in which enzymatically predigested casein is used in the hen's feed in order to observe the effect on the cystine and methionine content of the egg. Although enzymatically predigested casein is not completely hydrolyzed, sufficient quantities of free amino acids are present to minimize the factor of rate of absorption.

EXPERIMENTAL

Leghorn-Rhode Island crosses in their first year laying period (pullets) were used in these experiments.[2]

Three pullets were kept on feed similar to the types reported previously (Csonka et al., '47a): a low-protein basal diet in which corn meal furnished the main protein source (Csonka et al., '46a); a high-protein diet in which 20 parts of commercial casein (or predigested casein) were substituted for 20 parts of corn meal in the feed; and (Csonka et al., '46b) a high-protein diet in which 50 parts of soybean meal were substituted for 50 parts of corn meal.

In the experiment with predigested casein, the ration of one pullet was changed from laying mash to a low-protein (corn) ration. This was followed successively by a ration containing 20% predigested casein and then by a ration containing 20% casein, both representing a high-protein ration. In experiments with each of the other three specified diets, a pullet was changed from the laying mash to the test diet (table 1).

Three weeks after changing the diets the eggs were collected and used for analysis. The eggs (unfertile) were weighed at collecting time and stored at 35°C. until they were analyzed. The yolk was separated from the egg white and washed with a few milliliters of distilled water to remove the egg white quantitatively. Concentrated HCl in volume equal to that of the egg white volume was added to the egg

[2] The cooperation rendered by the Poultry Section of the Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, is gratefully acknowledged by the author.

TABLE 1

Distribution of nitrogen, cystine and methionine in the egg white (including membrane and shell) and yolk of eggs laid by hens fed varying proteins in feed

EXP. NO.	HEN NO.	HEN'S DIET	EGG LAID	EGG WHITE (PLUS MEMBRANE AND SHELL)			EGG YOLK		
				Nitrogen	Cystine	Methionine	Nitrogen	Cystine	Methionine
				mg	*mg*	*mg*	*mg*	*mg*	*mg*
1	2	Low-protein	11– 5–48	608	78	149	289	27	49
2		Soybean	12–10–48	631	83	145	379	32	48
3		Soybean	12–14–48	653	80	157	407	39	64
4		Casein	1– 6–49	596	81	158	437	42	65
5		Casein	2–16–49	668	75	152	407	38	62
6		Low-protein	4–28–49	482	64	125	402	38	59
6A		Low-protein	12– 5–49	332	70	121	367	58	48
7	3	Casein	11– 5–48	649	82	153	441	40	69
8		Low-protein	12–14–48	548	69	122	365	29	49
9		Soybean	2–28–49	707	75	158	467	43	66
10		Soybean	3– 9–49	679	68	155	513	55	75
11		Casein	4–24–49	703	74	148	494	51	90
12		Low-protein	6– 3–49	538	51	138	465	40	64
13	4	Low-protein	11– 2–48	631	81	171	320	25	49
14		Casein	2–17–49	724	82	176	487	47	72
15		Casein	3–18–49	718	60	151	473	38	65
16		Soybean	4– 7–49	706	63	160	507	44	70
17		Soybean	4– 8–49	651	63	157	510	46	75
18		Low-protein	6– 2–49	613	48	141	450	41	63

white, membrane, and shell. The egg shell and membrane were included in the egg white portion because they are also needed in the nutrition of the chick embryo for its full development. Ten milliliters of water and 30 ml of concentrated HCl were added to the yolk. Egg white and egg yolk were refluxed separately at the boiling point of HCl for 24 hours. The hydrolysates were then transferred to graduated cylinders, cooled to room temperature and the volumes noted. Usually the volume of yolk hydrolysate was between 55 and 60 ml and that of the egg white hydrolysate between 70 and 80 ml. The hydrolysates were then filtered. Aliquots of 5 ml from both hydrolysates were used for the estimation of cystine and methionine. Both aliquots were freed of excess HCl by being boiled down to a volume of from 1 to 1.5 ml. The samples were boiled twice again after the addition of 5 to 10 ml of distilled water. Finally, the egg white (w) and the egg yolk (y) samples were transferred into volumetric flasks and were made up with distilled water to the 25 ml mark. Ten milliliters of "W" were pipetted off for cystine determination and the remaining 15 ml served for methionine determination. The same procedure was followed with the "Y."

Five milliliters of 20% HCl were added to the methionine samples and the histidine was removed by tungstic acid. The procedure of determination employed was described in a previous publication (Csonka et al., '46a). The 10-ml aliquot used for the cystine determination was brought to pH 4.2, with glacial acetic acid and sodium hydroxide, and then the procedure was continued as described earlier (Csonka et al., '46b). The Beckman model DU spectrophotometer was used for color comparison.

The nitrogen content of the egg white and egg yolk hydrolysates was determined by the Kjeldahl method.

RESULTS AND DISCUSSION

The high-protein diet fed to laying hens increased the weight of the eggs (Csonka et al., '47a) and the nitrogen content of both egg white and egg yolk, as is shown in table 1.

Since the formation of yolk occurs during the 8 to 10 days prior to ovulation (by which time the ovum reaches its maximum size), while it takes approximately 4 hours for the secretion of the egg white, one might expect to find that the significant change in the composition of the egg brought about by feeding the hen rations in which the protein varies in kind and quantity would be produced in the yolk. We reported previously that no free cystine or methionine could be detected in egg white (Csonka et al. '47b). Increase in nitrogen content therefore represents chiefly protein nitrogen; nitrogenous substances of a non-protein nature like choline, for instance, are present in the egg in relatively small quantities.

The age of the pullet as a factor controlling the change in nitrogen quantities in the eggs must also be considered, for reasons discussed later. Increase in nitrogen, a result of feeding a high-protein diet, will occur mainly in the yolk (25%) in the case of a young pullet. At the same time, the egg-white-nitrogen increase is less than 10%. The older pullets showed just the reverse of the conditions stated above. These percentages were calculated on the averages given in table 2.

In individual experiments, the pullet on the high-protein casein diet showed an egg-yolk-nitrogen increase of 44% over the nitrogen values observed during the low protein feeding period, while an increase of 24% was noted when the pullet was on the high-protein-soybean diet. The changes in the nitrogen content of the egg whites varied by only 6% or less. The increases in cystine and methionine in the yolks, as a result of the high-protein casein feeding, were 38 and 40%, respectively. In the case of the high-protein-soybean feeding, however, only the cystine showed an increase, of 18%. These eggs were layed by young pullets in the months of November and December. (See experiments 1, 2, 7 and 8 in table 1.)

It is known that the weight of the yolk of a pullet egg increases in the first year of the laying period. Therefore, we can explain the higher nitrogen, cystine and methionine con-

TABLE 2

The distribution of nitrogen, cystine (Cy) and methionine (Me) in the egg white and egg yolk of the chicken egg as affected by the diet and by the aging of the laying pullet

REMARKS	EGG WHITE				EGG YOLK			
	N	Cy.	Me.	Cy./Me.	N	Cy.	Me.	Cy./Me.
	mg	*mg*	*mg*		*mg*	*mg*	*mg*	
Old pullets Low-protein diet Means of exp. 6, 12, 18, 6-A	491	58	131	0.44	421	44	59	0.75
Young pullets Low-protein diet Means of exp. 1, 8, 13	596	76	147	0.52	325	27	49	0.55
Young pullets High-protein diet Means of exp. 2, 3, 7	644	82	152	0.54	409	37	60	0.62
Old pullets High-protein diet Means of exp. 4, 5, 9, 10, 11, 14, 15, 16, 17	684	71	157	0.45	477	45	71	0.63

tent in the yolks of eggs laid by the older pullets during the low-protein feeding period (experiments 1, 6, 8, 12, 13 and 18 in table 2). This is apparently a normal physiological change, and it should always be considered when interpreting dietary protein influence on the amino acid composition of the yolk of an egg. The data in table 2 are rearranged so that the dietary effect on the egg composition produced by varying the quantities of protein in the feed may be distinguished from that caused by the aging of the pullet. The averages reported on the high-protein diet in table 2 are calculated both from the casein and soybean experiments, so as to furnish a greater number of samples for the statistical analysis. It may be stated, however, that if the data from these two experimental diets are averaged separately, the conclusions herein reached would be in no way altered.

When the young pullets were fed a high-protein diet, the nitrogen, cystine and methionine content generally increased in both egg white and egg yolk. But there is an indication that the cystine-methionine ratio in the egg white decreases with the aging of the pullet, regardless of the diets employed. An increase of the ratio in the yolk as a result of aging was found to be statistically significant only during the low-protein dietary regimen. This increased cystine/methionine ratio in the yolk, which is characteristic of the egg laid by the low-protein-fed older pullet, may be duplicated in the younger pullet's egg by feeding her a high-protein diet. Compared with the older pullet, the younger laying pullet requires protein for growth in addition to maintenance and the proper amino acids for egg protein synthesis. Consequently, a high-protein diet will more efficiently furnish all of these amino acid requirements, including those needed for growth. The cystine/methionine ratio in the yolks of eggs laid by older pullets on a high-protein feed is practically the same as that of eggs laid by older pullets on a low-protein diet, although the absolute nitrogen, cystine and methionine values are considerably higher.

It has been shown in a previous publication (Csonka et al., '47a) that the addition of free methionine to the hen's diet had no effect on the methionine or cystine content of the egg. It is possible that methionine was not a limiting amino acid in the synthesis of the egg proteins. One may also interpret this to be a result of the differences between the rate of absorption of the free amino acid and of those in peptide bondage.

TABLE 3

The cystine and methionine content of whole egg as influenced by changing no. 1 hen's diet from laying mash to the specified experimental diets

EGG NO.	HEN'S DIET	EGG LAID	CYSTINE	METHIONINE
			mg/egg	*mg/egg*
1	Low-protein	11– 3–48	129	236
2	Low-protein	11–15–48	119	209
3	Low-protein	11–17–48	123	190
4	Predigested casein	11–30–48	120	215
5	Predigested casein	12– 2–48	150	247
6	Predigested casein	12– 7–48	135	248
7	Predigested casein	12– 8–48	172	266
8	Predigested casein	12–10–48	167	266
9	Perdigested casein	12–11–48	145	254
10	Predigested casein	12–12–48	146	269
11	Predigested casein	12–15–48	167	240
12	High-protein casein	1– 11–49	146	250
13	High-protein casein	1– 12–49	157	255

The experimental data shown in table 3 demonstrate conclusively that the cystine and methionine content of the whole egg was lowered by changing the hen's diet from laying mash to a low-protein diet, and was elevated when the ration was followed successively by a predigested casein ration and a ration containing casein. Apparently when all the amino acid components required for protein synthesis by the bird are offered, either as predigested casein or as casein, they are equally well-utilized, and result in an increased cystine and methionine content of the egg.

SUMMARY

Chicken eggs were analyzed for the sulfur-containing amino acids and nitrogen.

As the pullets aged and were fed a low-protein diet, the nitrogen quantity decreased in the egg white and increased in the egg yolk. Aging of the pullet, in itself, was not an important factor when a high-protein diet was fed, since the younger as well as the older pullets showed an increased quantity of nitrogen in both egg white and egg yolk under these conditions. Changes shown by the nitrogen values were generally paralleled by similar changes in the cystine and methionine values. The cystine/methionine ratio in the egg white decreases with the aging of the pullet, regardless of the diets employed; the ratio increases in the egg yolk as a result of aging, but it is statistically significant only during the low-protein dietary regimen. Enzymatically digested casein in the hen's feed increases the cystine and methionine content of the egg to the same extent as does casein.

ACKNOWLEDGMENT

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VITAMIN E DEFICIENCY IN CHICKS

II. PLASMA XANTHOPHYLL LEVELS AND VITAMIN E DEFICIENCY SYMPTOMS [1]

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FOUR FIGURES

(Received for publication June 28, 1950)

The effects of certain dietary factors on the occurrence of vitamin E deficiency symptoms in chicks have been investigated by Zacharias, Goldhaber and Kinsey ('50; see p. 359). Evidence was obtained that the commercial chick diet (herein referred to as natural diet) used in these experiments contained a factor, other than vitamin E, which protected chicks from symptoms of vitamin E deficiency. In the course of measuring the plasma-tocopherol levels of chicks raised on various diets, data were also obtained concerning their plasma-xanthophyll levels.[3] It was noted that the chicks fed the vitamin E-deficient basal diet developed deficiency symptoms and had low plasma-xanthophyll levels, whereas chicks fed

[1] This work was supported by a grant from the Foundation for Vision, Boston, Mass., for the study of retrolental fibroplasia.

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[3] In the micromethod of Quaife et al. ('49) for the spectrophotometric determination of total tocopherols, a correction for carotenoids is obtained at 460 mμ. In reporting the values for the carotenoid pigments read at 460 mμ, no attempt has been made to calculate the actual pigment concentration. The quantities of pigment are presented as density units. Strain ('42) states: "Variations in the proportions and in the spectral absorption properties of the several xanthophylls make it impossible to calculate exactly the pigment concentration from colorimetric measurements."

the natural diet did not develop the symptoms and had high plasma-xanthophyll levels. The observation that the symptoms did not occur when the plasma contained appreciable amounts of xanthophyll suggested the possibility that this substance, which is the chief carotenoid pigment of the chicken (Palmer, '15; Palmer and Kempster, '19), might be a factor in the natural diet which protects the chick from vitamin E deficiency symptoms.

The present study is concerned with the possible protective action of xanthophyll against vitamin E deficiency symptoms and with the effects of various dietary factors on the plasma-xanthophyll concentration.

EXPERIMENTAL[4] AND RESULTS

Direct evidence for the protective action of xanthophyll

Oral supplements of crude xanthophyll[5] (120 mg per capsule) were given three times a week to chicks raised on the vitamin E-deficient basal diet. A total of 23 of 48 chicks (4 experiments) survived the entire experimental period (6 weeks) without developing vitamin E deficiency symptoms, although their plasma-tocopherol levels were no higher than those of a group of 35 control chicks raised on the unsupplemented basal diet. In the control group, 33 chicks developed deficiency symptoms during the third and 4th weeks.

Indirect evidence for the protective action of xanthophyll

A. In chicks raised on the basal diet (which was xanthophyll-free), the plasma-xanthophyll level decreased progressively, indicating that the xanthophyll stores were being depleted (fig. 1). Deficiency symptoms appeared in this group when the plasma-xanthophyll level had dropped to approximately 0.1 density units. On the other hand, when chicks were

[4] Except where otherwise indicated, all experimental procedures have been described in the previous study (Zacharias, Goldhaber and Kinsey, '50).

[5] Kindly supplied by the Midwest Extraction Company, Rockford, Illinois.

fed the natural diet (which contained xanthophyll), the plasma-xanthophyll level was maintained at approximately 0.5 density units or higher, and no deficiency symptoms occurred (fig. 1).

B. Chicks were raised on the natural diet to which various amounts of cod liver oil (5% and 20%) were added. Figure 2

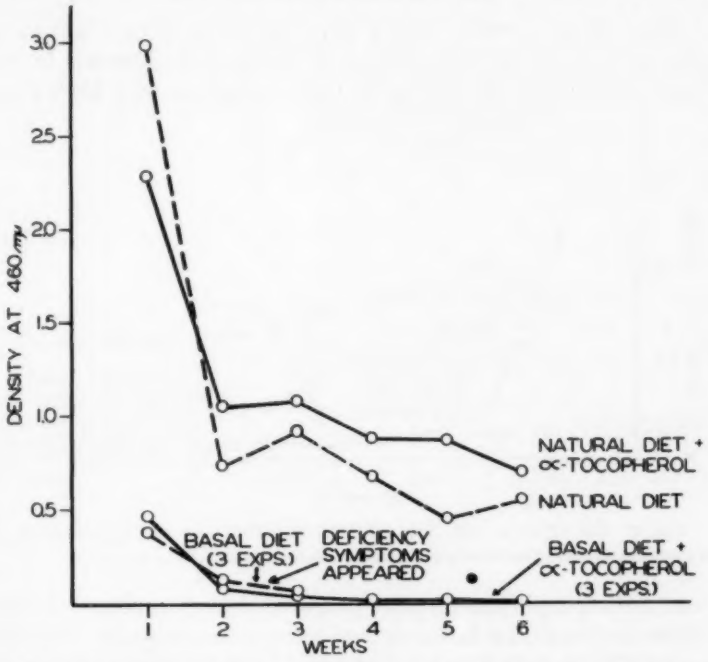

Fig. 1 The concentration of xanthophyll in the plasma of chicks raised on various diets. Each point represents the average concentration for an entire group. Chicks receiving the vitamin E-deficient basal diet could not be maintained after the third week.

shows that the plasma-xanthophyll level was lowered by the addition of cod liver oil to the natural diet, and that the degree of lowering seemed to be proportional to the amount of cod liver oil used. No deficiency symptoms occurred in the group fed the natural diet and 5% cod liver oil, whereas 11 of 18

chicks fed the natural diet and 20% cod liver oil developed symptoms. It should be noted that the symptoms appeared during the period when the plasma-xanthophyll level was approximately 0.1 density units.

The factors in cod liver oil which lower the plasma-xanthophyll level

Since Mattson and Deuel ('43), and Deuel et al. ('43) have demonstrated a decreased level of carotenoid pigments in the blood of chickens following the adminstration of a high vita-

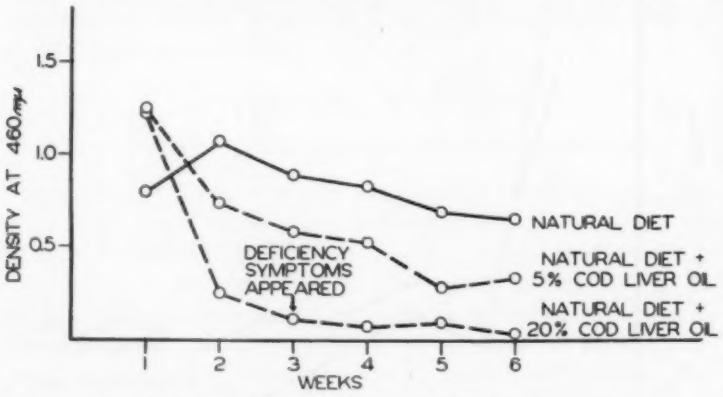

Fig. 2 The effect of cod liver oil (5% and 20%) on the concentration of xanthophyll in the plasma of chicks raised on the natural diet.

min A diet, experiments were performed to determine whether vitamin A was the factor in cod liver oil responsible for lowering the plasma-xanthophyll level. Chicks were reared on the following diets: (a) crude xanthophyll-supplemented basal diet (containing 5% cod liver oil); (b) crude xanthophyll-supplemented basal diet in which a water-miscible preparation of vitamins A and D [6] was substituted for 5% cod liver oil;

[6] Aquasol, which is an aqueous solution (U. S. Vitamin Corporation, New York, N. Y.) containing 1,000 units of natural vitamin A and 200 units of natural vitamin D per drop. The weekly dosage was as follows: first and second weeks, one drop; third and 4th weeks, two drops; 5th week, three drops; remainder of experiment, 4 drops weekly.

(c) crude xanthophyll-supplemented basal diet containing neither cod liver oil nor vitamins A and D.

Figure 3 shows that the plasma-xanthophyll level was decreased by the preparation containing vitamins A and D but

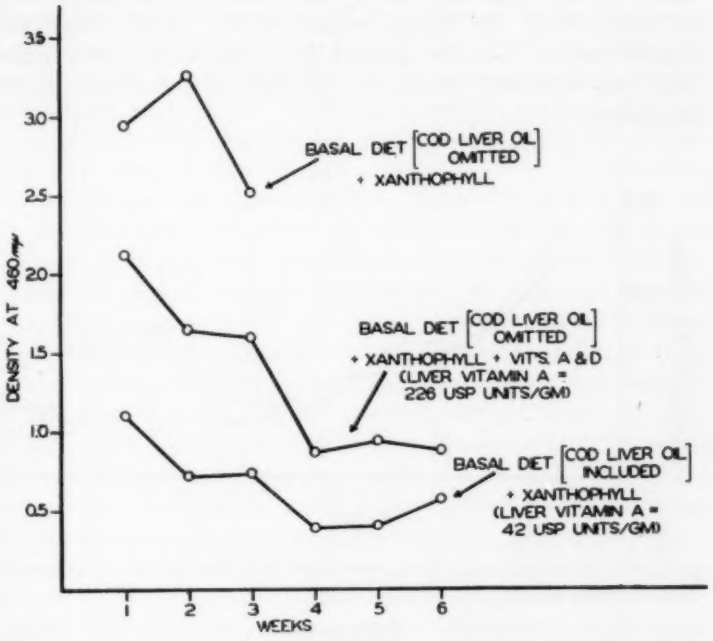

Fig. 3 The effect of cod liver oil and of vitamins A and D on the concentration of xanthophyll in the plasma of chicks raised on a xanthophyll-supplemented basal diet. The chicks fed the xanthophyll-supplemented basal diet from which the cod liver oil was omitted were sacrificed at the end of the third week of the experiment. These animals were in poor condition because of the absence of vitamins A and D in their diet.

to a lesser extent than by cod liver oil, despite the fact that assays of liver vitamin A showed that the liver vitamin A content was approximately 5 times greater in chicks fed vitamins A and D than in those receiving cod liver oil.

The effect of α-tocopherol on plasma-xanthophyll levels

Oral supplements of α-tocopherol were fed to chicks raised on the basal diet and on the natural diet. Figure 1 shows that the rate of loss of xanthophyll from the plasma of chicks raised on the basal diet was not affected by oral supplements of α-tocopherol. In chicks raised on the tocopherol-supplemented natural diet, the plasma-xanthophyll level was higher than that in chicks raised on the same diet without supplementation.

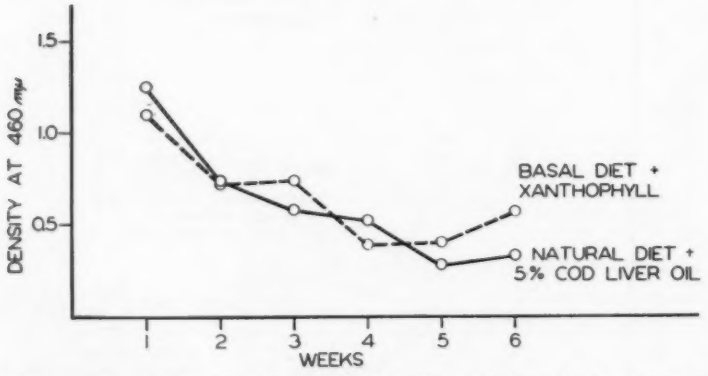

Fig. 4 The similarity of the concentration of xanthophyll in the plasma of chicks fed the xanthophyll-supplemented basal diet and the cod liver oil-supplemented natural diet.

DISCUSSION

Although the xanthophylls are widely distributed in plant and animal forms, only minor physiological functions have so far been assigned to them. For example, cryptoxanthin can be converted to vitamin A in the rat (Deuel et al., '45) and may possibly be utilized as vitamin A in the chicken (With, '46). In addition, the xanthophylls serve as color filters in the retina (Wald, '45). On the basis of the experimental results reported in this paper, it appears that xanthophyll can, in chicks, give partial protection against vitamin E deficiency symptoms. Approximately 50% of the chicks (23 of 48), raised on the

vitamin E-deficient basal diet, were protected from deficiency symptoms by supplements of crude xanthophyll. The plasma-xanthophyll levels of these chicks were approximately the same as those found in chicks fed the natural diet and 5% cod liver oil (fig. 4). In the latter group, however, none of the chicks developed symptoms. The difference in the degree of protection provided by the natural diet and the diet containing the xanthophyll supplements may have been caused by possible differences between the xanthophylls of the natural diet and those of the crude preparation used, or by additional protective factors in the natural diet.

Like tocopherol, xanthophyll is readily oxidized, and its protective action in preventing symptoms of vitamin E deficiency in chicks may result from its anti-oxidant activity. Quackenbush et al. ('42), Jensen et al. ('43) and Guggenheim ('44) have reported the sparing of carotene by tocopherol. Our experimental results have demonstrated a similar sparing of xanthophyll by tocopherol. This protection of xanthophyll appears to occur only in the gastrointestinal tract; the xanthophyll reserves of chicks raised on the xanthophyll-free diet were not protected by tocopherol (fig. 1). In addition, when a pro-oxidant (crystalline vitamin A) was fed to chicks raised on an α-tocopherol-supplemented natural diet, there was a decrease in the plasma-tocopherol level (Zacharias et al., '50), whereas the plasma-xanthophyll level was apparently unaffected. This observation suggests that tocopherol is preferentially oxidized and that xanthophyll is destroyed less rapidly in the presence of tocopherol. It appears, therefore, that xanthophyll protects chicks from vitamin E deficiency symptoms by a mechanism different from that of ascorbic acid, since ascorbic acid presumably protects the tocopherol of the tissues from oxidation (Dam et al., '48).

Some constituent of cod liver oil (probably the unsaturated fatty acids) appears to be necessary for the production of vitamin E deficiency symptoms in chicks. It has been found that the addition of cod liver oil to an α-tocopherol-supplemented diet caused a drop in the plasma-tocopherol level

(Zacharias et al., '50). If xanthophyll and tocopherol act similarly, one might also expect the plasma-xanthophyll concentration to decrease in the presence of cod liver oil. Figure 2 shows that the plasma-xanthophyll level was indeed lowered by cod liver oil. Moreover, the magnitude of the decrease was dependent upon the amount of cod liver oil fed. This figure also indicates that vitamin E deficiency symptoms appeared when the plasma-xanthophyll level had decreased to 0.1 density units, thus indicating a correlation between the destruction of xanthophyll by cod liver oil and the appearance of symptoms. It is of interest to note that the appearance of deficiency symptoms in chicks raised on the basal diet, and in those receiving the natural diet and 20% cod liver oil, occurred at a time when the plasma-xanthophyll had fallen to the same low level of 0.1 density units.

With regard to the factor in cod liver oil responsible for the lowering of the plasma-xanthophyll level, our experimental results indicate that some unknown factor (probably the unsaturated fatty acids) in cod liver oil, as well as vitamin A, contributes to the destruction of xanthophyll. This observation is not in full agreement with the findings of Rubin and Bird ('46), who have reported that the pigmentation-suppressing factor in fish liver oils is vitamin A.

Sherman ('47) reported that the addition of xanthophyll to the diet of rats decreased the gastrointestinal destruction of carotene, vitamin A alcohol, and vitamin A acetate. With regard to this observation, Sherman suggested that the protective effect of xanthophyll may be of practical significance in "certain human dietaries and animal feeds supplying vitamins A and E in suboptimum quantity." Our results appear to support this suggestion and indicate that a major function of xanthophyll may lie in its anti-oxidant activity.

SUMMARY

1. Oral supplements of crude xanthophyll prevented the appearance of deficiency symptoms in 23 of 48 chicks raised

on the vitamin E-deficient basal diet. This protection is ascribed to the anti-oxidant activity of the xanthophylls.

2. The addition of 20% cod liver oil to the natural diet caused a marked fall in the plasma-xanthophyll level. Vitamin E deficiency symptoms occurred in these animals when the plasma-xanthophyll level had decreased to 0.1 density units, thus indicating a correlation between the destruction of xanthophyll by cod liver oil and the appearance of symptoms.

3. Some unknown factor (probably the unsaturated fatty acids) in cod liver oil, as well as vitamin A, contributes to the destruction of xanthophyll.

4. The protection of xanthophyll by tocopherol occurs only in the gastrointestinal tract.

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NIACIN-REPLACING VALUE OF L- AND DL-TRYPTOPHAN IN CHICK DIETS AS INFLUENCED BY CARBOHYDRATE SOURCE [1]

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The ability of tryptophan to replace niacin in the diet of various animals has been the subject of many studies since the work of Krehl et al. ('45). In studies with chickens, Briggs ('45) found that the addition of 0.2% of DL-tryptophan to a niacin-low diet resulted in normal growth and prevented the appearance of niacin deficiency symptoms.

Subsequent studies indicate that this niacin-replacing action is due to the conversion of tryptophan to niacin. No reports have appeared concerning the comparative value of L- and DL-tryptophan in replacing niacin in the diet of chicks. However, studies have been reported in which the relative value of L- and DL-tryptophan was determined in diets which contained adequate niacin but were low in tryptophan. Grau and Almquist ('44) reported that the D-isomer was not used by the chick. Wilkening and Schweigert ('47) later found that from 17 to 40% of the D-tryptophan was utilized. The diet used by the latter workers contained corn starch as the source of carbohydrate whereas that used by the earlier workers contained glucose (cerelose). This suggested that the difference in utilization of D-tryptophan noted by the two groups might be due to differences in the bacterial utilization of tryptophan.

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In the present study L- and DL-tryptophan were compared as to their ability to replace niacin in the diet of the chick using either glucose or corn starch as the source of carbohydrate.

EXPERIMENTAL

Day-old New Hampshire chicks of both sexes were divided into uniform groups and reared in electrically heated batteries with raised wire floors. Feed and water were given ad libitum. Weighings and other observations were made weekly and all experiments were conducted for a 4-week period.

The basal ration (113N) used had the following percentage composition: crude casein 18, gelatin 10, salts 1 M (Briggs, '46) 6, soybean oil 4, DL-methionine 0.3, and glucose (cerelose) 61.4. Each 100 gm of diet also contained the following amounts of vitamins (in milligrams): thiamine hydrochloride 0.4, riboflavin 0.8, calcium pantothenate 2.0, pyridoxine hydrochloride 0.6, choline chloride 200, *i*-inositol 100, biotin 0.02, *p*-aminobenzoic acid 0.2, 2-methyl-1,4-naphthoquinone 0.1, and α-tocopherol acetate 0.5. Vitamins A and D_3 (1,200 U.S.P. units and 170 A.O.A.C. units, respectively) were fed by dropper weekly. When corn starch was used, it replaced all of the glucose of the diet. All additions were made at the expense of the carbohydrate. In the last experiment, sulfasuxidine was added at a level of 2% of the diet during the first two weeks, after which the level was reduced to 1%.

RESULTS

In the first two experiments graded levels of L- and DL-tryptophan were added to diet 113N, with glucose used as the source of carbohydrate. The results are given in table 1. This same diet was used in part of the third experiment and the results are given in table 2.

The mean gain for all groups of chicks fed L-tryptophan was significantly greater than for the groups of chicks fed an equal level of DL-tryptophan when glucose was used as the carbohydrate. The gain of the chicks fed L-tryptophan was greater than that of chicks fed twice the level of DL-tryptophan

TABLE 1

Comparative value of L- *and* DL-*tryptophan in replacing niacin in the diet of the chick (glucose in diet)*

GROUP NO.	SUPPLEMENT TO BASAL RATION 113N	EXPERIMENT 1		EXPERIMENT 2	
		No. chicks at finish [1]	Gain in 4 weeks	No. chicks at finish [1]	Gain in 4 weeks
			gm		*gm*
1	None	5	90	3	107
2	0.0125% L-tryptophan	..	..	3	47
3	0.025% DL-tryptophan	..	..	7	77
4	0.025% L-tryptophan	6	197	4	98
5	0.050% DL-tryptophan	6	165	8	82
6	0.050% L-tryptophan	6	201	8	190
7	0.10% DL-tryptophan	6	200	8	120
8	0.10% L-tryptophan	6	282	10	250
9	0.20% DL-tryptophan	6	275	10	234
10	0.40% DL-tryptophan	..	..	10	247
11	10 mg niacin/100 gm	6	274	10	247

[1] All groups in experiment 1 were started with 6 chicks; all groups in experiment 2 were started with 10 chicks.

TABLE 2

Effect of the type of dietary carbohydrate and of sulfasuxidine on the apparent value of D-*tryptophan*

GROUP NO.	SUPPLEMENT TO BASAL RATION 113N	GLUCOSE		STARCH	
		No. chicks at finish [1]	Gain in 4 weeks	No. chicks at finish [1]	Gain in 4 weeks
			gm		*gm*
Experiment 3 (No sulfasuxidine)					
1	None	6	88	8	48
2	0.025% L-tryptophan	13	101	15	71
3	0.050% DL-tryptophan	13	128	11	114
4	0.050% L-tryptophan	13	145	9	107
5	0.10% DL-tryptophan	16	171	15	148
6	10 mg niacin/100 gm	10	257	10	270
Experiment 4 (With sulfasuxidine)					
1	None	8	120	9	94
2	0.025% DL-tryptophan	15	137	10	68
3	0.025% L-tryptophan	15	197	14	94
4	0.050% DL-tryptophan	15	187	14	103
5	0.050% L-tryptophan	15	206	11	175
6	0.10% DL-tryptophan	14	192	15	194
7	10 mg niacin/100 gm	15	332	14	307

[1] All groups in experiment 3 were started with 17 chicks except groups 1 and 6, which had 10; all groups in experiment 4 were started with 15 chicks.

in 6 of the 9 comparisons. Considering all groups, regardless of the level of tryptophan fed, the chicks fed L-tryptophan grew slightly faster than those receiving twice the level of DL-tryptophan. The difference, however, was not significant. Since a given level of L-tryptophan was approximately equal in value to twice the level of DL-tryptophan, only the L-isomer appeared to be used for conversion to niacin when glucose was fed as the source of carbohydrate.

When corn starch was used as the source of carbohydrate, a given level of DL-tryptophan promoted greater growth than L-tryptophan fed at one-half the level, both in the presence or absence of sulfasuxidine. In experiment 3, when starch was used as the source of carbohydrate, the addition of 0.05% DL-tryptophan promoted as great a growth response as did 0.05% L-tryptophan. When groups 2 and 4 are compared with groups 3 and 5, the difference in average weight gain is significant. This indicates that when starch was used as the source of carbohydrate, the D-tryptophan was of value. Its value appeared to be decreased by the addition of sulfasuxidine to the diet, although the exact relationship cannot be determined from the data.

The results of two experiments designed to determine the effect of the type of carbohydrate and of sulfasuxidine on the conversion are given in table 2. Chicks fed the diets with glucose and tryptophan gained more weight than those fed diets containing starch and the same levels of tryptophan in every case except group 6 of experiment 4. The difference between the mean gains of the glucose- and the starch-fed chicks in both experiments were significant. The gains of the chicks fed sulfasuxidine were significantly greater than the gains of those not fed sulfasuxidine. The two experiments were not done simultaneously. However, the chicks fed the diet with sulfasuxidine, niacin, and glucose gained more than has been noted in approximately 30 experiments in which the same diet without sulfasuxidine has been fed. Moore et al. ('46) also noted similar differences when sulfasuxidine or streptomycin was fed. They suggested that sulfasuxidine may in-

hibit the growth of intestinal bacteria which are capable of producing toxic materials or of rendering certain dietary vitamins unavailable to the animal.

In these experiments wide variation was noted in the ability of individual chicks to grow with the suboptimum levels of niacin and tryptophan fed. Individual chicks were observed to attain weights of from 200 to 300 gm and to show no niacin deficiency symptoms when fed the basal diet. In experiments 2 and 4, such chicks formed a large part of the surviving chicks of the groups fed the basal diet, with the result that the group-mean gains for these groups were greater than those for groups fed the low levels of tryptophan.

Throughout each experiment the feed was used more efficiently by the more rapidly growing chicks. The percentage of chicks showing niacin deficiency symptoms was decreased as the level of tryptophan in the diet was increased.

DISCUSSION

These results indicate that D-tryptophan was utilized to a greater extent when the diet contained starch than when it contained glucose. It is believed that this difference is not due to a change in the chick's ability to utilize D-tryptophan but rather to a change in the bacterial flora of the intestinal tract resulting from the use of starch in place of glucose. Dann and Handler ('41), Snell and Quarles ('41) and Levy and Young ('48) have reported that the total amount of niacin in the egg increases during the incubation of the egg. Furthermore, Schweigert et al. ('48) found that the addition of DL-tryptophan to the egg resulted in an increase in the niacin content of the egg later in incubation. These findings demonstrate that the chick tissues can synthesize niacin from tryptophan. The change from glucose to starch in the diet should make no difference in the ability of chick tissues to utilize D-tryptophan. Since the experiments in which glucose was used indicate that L-tryptophan is approximately twice as active as DL-tryptophan, it is believed that the chick itself uses only the L-tryptophan for the conversion.

The apparent value of the D-tryptophan may result either from bacterial conversion to L-tryptophan, niacin, or any substance which the chick could convert to niacin, or by D-tryptophan's sparing the amount of L-tryptophan made unavailable to the chick by bacterial action. The apparent value of the D-tryptophan noted in experiments 3 and 4, with starch in the diet, appears to be a result of both of these actions. In both experiments the chicks fed tryptophan and starch grew at a slower rate than those fed the same levels of tryptophan and glucose. However, these differences were not as apparent when niacin was added to the diet. This suggests that more tryptophan is utilized by bacteria in the digestive tracts of the chicks fed starch than in those of the chicks fed glucose.

It is possible that feeding sulfasuxidine decreases the bacterial utilization of tryptophan and that this may be the reason why the chicks which were not fed niacin in experiment 4 gained more than the chicks fed the same diets without sulfasuxidine in experiment 3. However, Gant et al. ('43) and Light et al. ('42) found that the total number of bacteria was not reduced by feeding sulfasuxidine or sulfaguanidine. The increase in growth which was noted when sulfasuxidine was added to the diet containing niacin cannot be due to a decrease in bacterial use of the tryptophan which would otherwise be available for conversion to niacin. Evidently, feeding sulfasuxidine decreases bacterial use of other nutrients or stimulates the synthesis of unknown factors or, in these experiments, possibly the synthesis of vitamin B_{12}. Vitamin B_{12} was not added to these diets. However, the diets contained crude casein and the chicks were from hens which were fed animal proteins. Stokstad and Jukes ('50) found that increased growth resulted when aureomycin, sulfasuxidine, streptomycin, or 3-nitro-4-hydroxyphenylarsonic acid was added to chick diets containing adequate levels of the known required nutrients, including vitamin B_{12}.

Intestinal bacteria are known to affect the synthesis of niacin. Ellinger et al. ('44) and Ellinger et al. ('45) found that humans given sulfasuxidine excreted, on the average,

60% less nicotinamide methochloride in the urine than those not given this drug. They concluded that the decrease was due to decreased bacterial synthesis of niacin. Feeding sulfasuxidine to rats also decreased the excretion of nicotinamide methochloride after tryptophan was fed (Ellinger and Abdel Kader, '47, '49). These workers found that the coliform bacteria were reduced in number by feeding sulfasuxidine; ambamide (*p*-aminomethylbenzenesulphonamide) was found to increase both the number of coliforms in the intestinal flora and the urinary excretion of nicotinamide methochloride (Ellinger and Emmanuelowa, '46; Ellinger et al., '47). Ellinger and his associates have suggested that the coliform bacteria are primarily responsible for the conversion. Moore et al. ('46) also found that the coliforms were reduced in number in the cecal contents of chicks fed sulfasuxidine. In the studies presently reported, the D-tryptophan appeared to have more value when sulfasuxidine was not fed, which is consistent with these observations.

In the experiments of Grau and Almquist ('44) and Wilkening and Schweigert ('47), sufficient niacin was added to supply the chicks' needs. The tryptophan, theoretically, was used per se in building protein. If chick tissues have the ability to utilize D-tryptophan for any essential purpose, it should have been reflected in our experiments in which the diets contained glucose. Since it was not, it seems probable that the utilization of D-tryptophan noted by Wilkening and Schweigert resulted from bacterial action, since corn starch was used in the diet.

The apparent value of the D-isomer that was noted in this study is probably not limited to the amino acid, tryptophan. The unnatural isomer of other amino acids may be utilized by certain intestinal bacteria, while the isomer as such would be of no value to the host animal. The dietary requirement for other amino acids may be influenced by the type of carbohydrate in the diet. The use of a ration containing glucose in determining amino acid requirements may indicate a requirement different from that which would result if natural feedstuffs were used.

SUMMARY

L- and DL-tryptophan were compared with respect to their ability to replace niacin in the diet of the chick. The L-isomer appeared to be the only one utilized by chick tissues. This isomer was found to be approximately twice as active as DL-tryptophan when the diet contained glucose as the source of carbohydrate. When starch replaced glucose in the diet, the D-tryptophan was of some value. The difference in value of the D-tryptophan when starch was substituted in place of glucose is believed to be the result of a change in the bacterial flora of the digestive tract. The value of the D-tryptophan was decreased by the addition of sulfasuxidine to the diet.

Replacing the glucose of the diet by starch decreased the growth rate except when niacin was added. Chicks fed niacin-adequate diets containing added sulfasuxidine grew more rapidly than those fed the same diet without sulfasuxidine.

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VITAMIN B_{12}, APF CONCENTRATES, DRIED WHEY, FISH SOLUBLES AND LIVER FRACTION "L" IN THE NUTRITION OF THE MATURE FOWL [1, 2]

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Lillie, Olsen and Bird ('49) presented data which indicated the importance of vitamin B_{12} for reproduction in poultry. Olcese, Couch and Lyman ('50a) reported that the hatchability of eggs from hens fed a diet low in B_{12} decreased to zero in from two to 4 weeks and, further, that the hatchability of eggs from hens fed this diet, supplemented with animal protein factor (APF) concentrates, decreased about the 9th to 10th week of the experimental period; which indicated that the birds had been depleted of a second factor, since the APF concentrates were supplying B_{12}. In a later study, Olcese and Couch ('50) reported that the injection of crystalline vitamin B_{12} into eggs from hens fed a diet low in B_{12} promoted normal hatchability from the 6th through

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the 8th week, but failed to do so after the 9th week in a 17-week experiment. Injection of vitamin B_{12} into deficient eggs (Olcese and Couch, '50) prevented the occurrence of B_{12} deficiency symptoms in the embryo described by Olcese et al. ('50b) throughout the 17-week test. Cravens and Halpin ('49) stated that an unidentified factor is required by the hen for egg production and hatchability, and that liver "L" and fish solubles were good sources of this factor.

Since the isolation of vitamin B_{12} (Rickes et al., '48), the pure vitamin has been reported by several groups of workers to promote growth responses in chicks comparable to those obtained with crude sources of the animal protein factor (Ott, Rickes and Wood, '48; Nichol et al., '49). More recent reports indicate that the animal protein factor complex is composed of unidentified factors in addition to vitamin B_{12}, and that such unidentified factors are required in addition to B_{12} for maximum chick growth (Carlson et al., '49; Menge, Combs and Shorb, '49; Stokstad et al., '49; Combs and co-workers, '50; Sunde et al., '50).

The present study was designed to determine whether the hen requires factors other than vitamin B_{12} for egg production and hatchability, and to study the effect of supplementing a diet low in vitamin B_{12} with possible crude sources of such factors.

MATERIALS AND METHODS

The Single-Comb White Leghorn pullets used had been reared on range and were placed in individual laying cages at approximately 5 months of age. After a pre-experimental period of 6 weeks, during which time individual egg production and hatchability records were kept, 16 groups of 4 birds each were selected for the experiment herein reported. The management and experimental procedures with regard to the care and handling of the birds, insemination, incubation and embryonic mortality records were the same as those previously reported by Couch et al. ('48) and Olcese, Couch and Lyman ('50a).

The composition of the basal diet (A-1) was the same as that employed by Olcese, Couch and Lyman ('50a) and consisted of 68% sucrose, 22% soybean protein (type M),[3] 3% soybean oil, 2% fortified fish oil (3,000 A–400 D) and 5% salts IV. The following amounts of vitamins were added in milligrams per kilogram: thiamine hydrochloride, 4; riboflavin, 6; calcium pantothenate, 15; niacin, 100; pyridoxine hydrochloride, 4; 2 methyl-1,4-naphthoquinone, 0.5; alpha-tocopherol, 3; folacin (PGA), 2; biotin, 0.2; choline chloride, 2,000; PABA, 20, and inositol, 1,000. In addition, 7.5 gm methionine and 4 gm glycine were added per kilogram of diet.

The APF 3 was a fuller's earth adsorbate of vitamin B_{12} [4] standardized to contain approximately 27.5 μg B_{12} per gram; animal protein factor concentrate 4,[5] was found by microbiological assay to contain 0.6 μg B_{12} per gram. The liver fraction "L" was found to contain approximately 1.24 μg B_{12} per gram, and the fish solubles contained 0.6 μg B_{12} per gram by microbiological assay. This B_{12} content of fish solubles is higher than that reported by Lewis et al. ('49), where a rat growth assay procedure was used to determine B_{12}. The dried whey was found to contain 0.025 μg B_{12} per gram by microbiological assay.

The above-mentioned values were obtained according to the microbiological assay procedure of Skeggs et al. ('48) with *Lactobacillus leichmannii 4797* (A.T.C.C.) as the test organism. Thiomalic acid was used in the medium as a growth factor for *L. leichmannii,* as a reducing agent, and for the protection of B_{12} during autoclaving. Acid production was the criterion used to measure the growth of the microorganism.

The first group of 4 pullets was fed the basal diet (A-1) unsupplemented. The second group was fed diet A-1 and 2 μg of crystalline B_{12} in 0.2 ml of water were injected into the pectoral muscle of each bird weekly. The third group was fed diet A-1 and each bird was fed 10 μg of crystalline B_{12} in

[3] Buckeye Cotton Oil Company, Cincinnati, Ohio.

[4] Supplied by Merck and Company.

[5] Supplied by the Lederle Laboratories (8108–109).

1 ml of water weekly. The 4th and 5th groups were fed diet A-1, supplemented with 2½ (69 μg B_{12}) and 5 gm (138 μg B_{12}) APF 3 per kilogram, respectively. Group 6 was fed 50 gm (1.3 μg B_{12}) dried whey substituted for an equivalent amount of sucrose in diet A-1. Group 7 was fed diet A-1, supplemented with 5 gm APF 3 and 50 gm dried whey per kilogram of diet. Groups 8, 9, and 10 were fed diet A-1, supplemented with 10 (6 μg B_{12}), 20 (12 μg B_{12}) and 50 (30 μg B_{12}) gm APF 4 per kilogram of diet, respectively. Group 11 received 20 gm APF 4 and 50 gm dried whey per kilogram as supplements to diet A-1. Group 12 was fed 40 gm fish solubles (24 μg B_{12}) per kilogram of diet substituted for an equivalent amount of sucrose in diet A-1. Group 13 received 20 gm APF 4 and 40 gm fish solubles per kilogram of diet as supplements to diet A-1. In the case of group 14, 40 gm liver fraction "L" (49.6 μg B_{12}) were substituted for sucrose in diet A-1. Group 15 was fed the basal diet (A-1) and each bird received a weekly injection of 0.1 ml liver extract,[6] which was equivalent to 2 μg B_{12}. All of the above-mentioned supplements to the basal diet were added in place of an equivalent amount of sucrose. The remaining 4 hens in this experiment (group 16) were fed a practical all-mash diet which contained: 42% ground yellow corn, 15% ground oats, 10% wheat bran, 10% wheat gray shorts, 2% meat and bone scraps, 5% alfalfa leaf meal, 2% fish meal, 12% soybean oil meal, 1% bone meal, ½% salt and ½% fish oil. Ten grams of manganese sulphate and 20 gm BY-21[7] were added per 100 pounds.

All experimental diets were mixed fresh once each week and were kept before the birds at all times. Oyster shell and tap water were supplied ad libitum.

RESULTS AND DISCUSSION

Egg production

The results of this study indicate that vitamin B_{12} is required for egg production. The per cent production of hens

[6] Lederle.

[7] Source of riboflavin. Dried fermentation solubles containing 8,000 μg of riboflavin per gram.

injected with 2 μg of vitamin B_{12} per bird per week (group 2) was higher than that of birds fed diet A-1 unsupplemented (group 1, table 1). The weekly oral feeding of vitamin B_{12}

TABLE 1

Effect of vitamin B_{12}, APF concentrates, dried whey, fish solubles, liver "L" and liver extract on egg production

GROUP NUMBER	SUPPLEMENTS TO BASAL DIET A-1	WEEKS				FOR PERIOD
		0–4	5–8	9–12	13–16	
	gm/kg	%	%	%	%	%
				Egg production		
1	None	11	37	63	22	33
2	2 μg B_{12} injected per hen per week	50	17	73	70	52
3	10 μg B_{12} orally per hen per week	23	22	59	52	39
4	2½ gm (69 μg B_{12})[1] APF 3	22	47	60	58	46
5	5 gm (138 μg B_{12})[1] APF 3	35	43	68	77	52
6	50 gm dried whey (1.3 μg B_{12})[1]	50	75	58	69	62
7	5 gm APF 3 and 50 gm dried whey	49	56	64	67	59
8	10 gm (6 μg B_{12})[1] APF 4	46	52	70	Discontinued	53
9	20 gm (12 μg B_{12})[1] APF 4	57	56	63	42	55
10	50 gm (30 μg B_{12})[1] APF 4	48	62	84	58	60
11	20 gm APF 4 and 50 gm dried whey	56	58	75	71	65
12	40 gm fish solubles (24 μg B_{12})[1]	46	39	61	59	51
13	20 gm APF 4 and 40 gm fish solubles	37	32	60	38	42
14	40 gm liver "L" (49.6 μg B_{12})[1]	48	49	74	73	60
15	0.1 ml (2 μg B_{12}) liver extract injected per hen per week	53	50	65	67	58
16	Practical	62	61	74	79	69

[1] Approximate micrograms B_{12} per kilogram of diet.

(10 μg per bird per week) produced an increase in the per cent egg production over that of pullets fed diet A-1 (group 1), but oral feeding of the vitamin was less effective in increasing egg production than was a weekly injection of B_{12} (groups 2 and 3, table 1). The poorer egg production which resulted from oral feeding of vitamin B_{12} as compared to that resulting from the injection of the vitamin may have been due to a number of factors. The quantity fed orally may have been too small (equivalent to about 10 μg B_{12} per kilogram of diet, since the food consumption of the hens was approximately 1 kg per bird per week). Better results might have been obtained had the crystalline B_{12}, which was fed orally, been administered more often than once weekly.

The feeding of APF 3 (groups 4 and 5) similarly produced an increase in the per cent egg production over that of pullets fed diet A-1 (table 1) but this was not greater, in any instance, than that of those injected with the crystalline vitamin. Olcese, Couch and Lyman ('50a) presented data which showed that vitamin B_{12} was required for egg production, although the tests reported by these workers were of shorter duration than those of the present study.

The results of this experiment show that dried whey may contain a factor which promotes egg production in the laying hen. The supplementation of diet A-1 with 5% dried whey (group 6) produced an increase in the per cent production over that of pullets injected with crystalline B_{12} (group 2, table 1), and likewise produced such an increase when the whey was fed with 5 gm APF 3 (group 7). The egg production of the birds fed APF 4 and dried whey (group 11) was higher than that of those fed APF 4 only (group 9). Attention is directed to the fact that the dried whey supplied only 1.3 μg of B_{12} per kilogram of diet.

The feeding of APF 4 (groups 8 and 9) produced a per cent egg production which was greater than that of the hens fed diet A-1 unsupplemented (group 1) and about the same as that of those injected with crystalline vitamin B_{12} (group 2). The per cent egg production of the birds fed 50 gm of

APF 4 per kilogram of diet (group 10) was increased above that of those injected with B_{12}. The above data indicate that the high level of APF 4 fed (50 gm per kilogram) may have provided the fowls with an additional factor required for egg production. Stokstad et al. ('49) reported that APF concentrates such as APF 4 contained a factor which increased chick growth over that observed when crystalline vitamin B_{12} was injected.

The increase in egg production associated with the feeding of 40 gm of fish solubles per kilogram of diet (group 12) over that of birds fed the basal diet (A-1) is thought to have been due to the vitamin B_{12} contributed by the fish solubles (24 μg B_{12} per kilogram).

Liver fraction "L" [8] and liver extract [9] (groups 14 and 15) increased the egg production of pullets over that of those injected with vitamin B_{12} (group 2). It is interesting to note that the per cent egg production of birds fed dried whey (groups 6, 7 and 11) and 50 gm of APF 4 (group 10) was higher than that of any of the other groups, with the exception of the birds fed the practical all-mash diet (group 16). The per cent egg production of the birds in group 16 was good throughout the test and was somewhat higher than that of other groups fed dried whey, 50 gm of APF 4 per kilogram (group 10) or liver "L." It is fully realized that the above-mentioned data were not treated statistically; yet the differences discussed do indicate quite definite trends. The egg production of the hens fed the diet low in B_{12} (A-1) was increased by injecting the crystalline vitamin or by feeding the APF concentrates which supplied B_{12}, and was further increased by feeding dried whey either alone or in combination with an APF concentrate.

Hatchability

The evidence obtained in the present investigation points to the fact that unidentified factors over and above vita-

[8] Wilson and Company.
[9] See footnote 6, page 476.

min B_{12} are required by the laying hen to produce satisfactory hatchability and normal embryonic development. The hatchability of eggs from hens fed diet A-1 unsupplemented (group 1) was less than 50% during the first 4 weeks of the test period and less than 5% from the 5th through the 12th week. There is little question but that the weekly injections of crystalline B_{12} (group 2) improved the hatchability of the eggs from these birds over that of eggs from those fed diet A-1 (table 2). The hatchability of eggs from hens fed B_{12} orally (group 3) was quite variable; it appeared to be normal during the first 4-week period, decreased decidedly during the second 4-week period, was normal from the 9th through the 12th week, and low again during the last 4 weeks of the experiment (table 2). The inadequacies of the level of B_{12} fed orally, the fact that it might have been better had the vitamin been administered more often, and the possible destruction of the vitamin in the gastrointestinal tract are all factors to be considered in evaluating the results obtained from the hens in group 3.

The injection of vitamin B_{12} (group 2), the feeding of APF 3 (groups 4 and 5) and the feeding of 20 gm of APF 4 per kilogram (group 9) produced satisfactory hatchability for the first 12 weeks of the test, but a decrease in the hatchability of eggs from all of these groups was noted from the 13th through the 16th week. Such a decrease was not noted in the hatchability of the eggs from hens fed liver fraction "L" (group 14, table 2). From these data it is apparent that the pullets used in this experiment were depleted of a second factor after about the 12th week, and that liver "L" contained this factor since this material supported a per cent hatchability of 96 from the 13th through the 16th week of the test.

The per cent hatchability of eggs from hens fed 40 gm fish solubles per kilogram of diet (group 12) and of eggs from hens fed fish solubles and 20 gm APF 4 per kilogram (group 13) decreased very decidedly after the 8th week of the test period. From these data it is evident that this sample of fish

TABLE 2

Effect of vitamin B_{12}, APF concentrates, dried whey, fish solubles, liver "L" and liver extract on per cent hatchability

GROUP NUMBER	SUPPLEMENTS TO BASAL DIET A-1	WEEKS			
		0–4	5–8	9–12	13–16
	gm/kg	%	%	%	%
				Hatchability	
1	None	43	4	4	0
2	2 μg B_{12} injected per hen per week	83	71	79	45
3	10 μg B_{12} orally per hen per week	75	37	87	34
4	2½ gm (69 μg B_{12})[1] APF 3	85	89	92	55
5	5 gm (138 μg B_{12})[1] APF 3	97	93	88	52
6	50 gm dried whey (1.3 μg B_{12})[1]	52	12	3	0
7	5 gm APF 3 and 50 gm dried whey	82	92	82	69
8	10 gm (6 μg B_{12})[1] APF 4	83	100	74	Discontinued
9	20 gm (12 μg B_{12})[1] APF 4	86	87	87	47
10	50 gm (30 μg B_{12})[1] APF 4	88	84	97	80
11	20 gm APF 4 and 50 gm dried whey	90	96	91	85
12	40 gm fish solubles (24 μg B_{12})[1]	84	74	40	45
13	20 gm APF 4 and 40 gm fish solubles	77	73	39	40
14	40 gm liver "L" (49.6 μg B_{12})[1]	78	82	90	96
15	0.1 ml (2 μg B_{12}) liver extract injected per hen per week	88	92	100	52
16	Practical	87	98	91	92

[1] Approximate micrograms B_{12} per kilogram of diet.

solubles did not contain the hatchability factor which was found in the liver fraction "L."

The per cent hatchability of eggs from hens fed dried whey (group 6, table 2) was approximately the same as that of those from hens fed diet A-1 unsupplemented. Dried whey (5%) supplied only 1.3 μg B_{12} per kilogram of diet. It is interesting to note that the per cent hatchability of eggs from hens fed dried whey and APF 3 (group 7, table 2), as well as that of those from hens fed whey and APF 4 (group 11), increased over that of eggs from hens injected with vitamin B_{12} during the last 4-week period of the experiment. This may mean that the dried whey contains the hatchability factor which becomes evident only when a source of B_{12} is in the diet, or that the whey stimulates the intestinal synthesis of the factor.

It is readily apparent that the per cent hatchability of eggs from hens fed 50 gm of APF 4 per kilogram (group 10) was higher than that of those from hens injected with crystalline vitamin B_{12} during the last 4 weeks of the test period. These data indicate that APF 4 contains a small amount of the hatchability factor but that it is necessary to feed a high level of the concentrate in order to show the effect of this APF on hatchability.

The liver extract used in the present investigation apparently did not serve as a source of the liver "L" hatchability factor, since the hatchability of the eggs from hens in group 15 decreased to about 50% during the last 4 weeks of the test. The hatchability results discussed above agree with the earlier reports of Olcese, Couch and Lyman ('50a) and Olcese and Couch ('50), in that hens fed a diet low in vitamin B_{12} (A-1) are depleted of a second factor at 9 to 12 weeks and that vitamin B_{12} alone will no longer support normal hatchability after the above-mentioned time. The present study provides additional information to the effect that liver fraction "L" is a source of the hatchability factor. It was indicated that dried whey fed with a source of vitamin B_{12} in the form of an APF concentrate and also a high level

of APF 4 provided lesser amounts of the liver "L" hatchability factor. The question arises as to whether the unidentified factor reported by Cravens and Halpin ('49) as being required for egg production and hatchability is the same as the liver "L" hatchability factor of the present investigation. The factor of Cravens and Halpin was found in liver "L" and fish solubles, whereas the sample of fish solubles used in the present study did not appear to contain the liver "L" hatchability factor. It should also be pointed out that the work of Cravens and Halpin was carried out before crystalline vitamin B_{12} was available for investigational use.

The occurrence of a factor necessary for egg production, and to a lesser extent of the liver "L" hatchability factor in dried whey, calls to mind the work of Hill et al. ('44) and of Hill ('48). The latter work shows that dried whey contains a factor which is necessary for chick growth and that such a factor is present in varying amounts in soybean oil meal. The present study shows that the factors in dried whey and liver fraction "L" are definitely necessary for egg production and hatchability. Other reports (Skinner et al., '50; Weise et al., '50; Yacowitz et al., '50) show that vitamin B_{12} or APF concentrates containing vitamin B_{12} will produce satisfactory egg production and hatchability when added to a soybean oil meal-corn diet, and the indications from this work are that additional factors over and above vitamin B_{12} are not needed. The evidence of the present investigation, which shows the existence of unidentified factors necessary for egg production and hatchability which are found in liver fraction "L" and dried whey, is not in contradiction to the work mentioned above, since these factors may either be present in soybean oil meal in sufficient quantities to meet the hen requirements or may be produced by bacteria in the gastrointestinal tract and be made available to the host animal.

SUMMARY

From the results of this study it is quite apparent that vitamin B_{12} is required for egg production in the domestic fowl.

Egg production was improved by feeding dried whey and liver fraction "L," by the injection of liver extract and by the feeding of a high level (5%) of an APF concentrate which is produced as a by-product in the manufacture of aureomycin. The above-mentioned products were therefore assumed to contain an unidentified factor which is required for egg production.

The hatchability of eggs from hens fed the basal diet (soybean protein-sucrose) decreased decidedly during the first 4 weeks of the test and few chicks hatched after this time. The addition of dried whey alone failed to improve hatchability appreciably.

The hatchability of eggs from hens injected with crystalline B_{12} and of those from hens fed APF concentrates decreased decidedly after the 12th week of the test, indicating that the hens were depleted of a second factor over and above vitamin B_{12} which is required for hatchability after the 12th week.

The feeding of liver fraction "L" maintained hatchability throughout the test period, and this product is believed to contain a factor necessary for hatchability in addition to vitamin B_{12}. The presence of lesser amounts of this factor in other materials was demonstrated by feeding dried whey with APF concentrates and by feeding a high level (5%) of an APF concentrate which is produced as a by-product in the manufacture of aureomycin.

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BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1951 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1951. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1951. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

HAZEL K. STIEBELING
Bureau of Human Nutrition and Home Economics
U. S. Department of Agriculture
Washington, D. C.

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1951 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Jury of Award of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1951.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Jury of Award, nominations for this award for work published in 1950 must be in the hands of the Chairman of the Nominating Committee by January 1, 1951. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Jury of Award in its consideration of the nomination.

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CHAIRMAN, NOMINATING COMMITTEE

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1951 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1951.

ROBERT V. BOUCHER
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CHAIRMAN, NOMINATING COMMITTEE